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(54) Title: CATIONIC COMPLEXES OF POLYMER–MODIFIED ADENOVIRUS

(57) Abstract

An adenovirus complex including a complex of a cationic molecule and of an adenovirus having at least one polyalkalene glycol polymer bound thereto. The polyalkalene glycol polymer includes, but is not limited to, polyethylene glycol, methoxypolyethylene glycol, polymethylethylene glycol, polyhydroxypropylene glycol, polypropylene glycol, and polymethylpropylene glycol. Molecular weights for the polymer range from 200 to 20,000 Daltons, with 2000 to 12,000 Daltons being preferred. The adenovirus is a preferably recombinant adenoviral vector such as a recombinant viral vector containing a transgene. The polymer is bound, directly or indirectly, to the virus particle by covalent or noncovalent means. The cationic molecule is preferably a cationic polymer, such as DEAE–Dextran, or a cationic lipid. A composition containing the adenovirus complex and a carrier is also disclosed.

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CATIONIC COMPLEXES OF POLYMER-MODIFIED ADENOVIRUS

SPECIFICATION

BACKGROUND OF THE INVENTION

Effective use of transgenes for the treatment of inherited and acquired disorders requires efficient delivery of transgenes. Various vector systems have been developed that are capable of delivering a transgene to a target cell. While newer generations of vectors having improved characteristics have been developed, there still remains a need to improve efficiency of available gene transfer methods. Improved efficiency is desirable both to increase the ability of the vector to deliver the transgene to target cells to correct the cellular defect, or provide a gene encoding a desirable product and to decrease the required amount of the vector and thereby reduce toxicity, including immunogenicity.

Adenoviral vectors, have been designed to take advantage of the desirable features of adenovirus which render it a suitable vehicle for nucleic acid transfer. Adenovirus is a non-enveloped, nuclear DNA virus with a genome of about 36 kb, which has been well-characterized through studies in classical genetics and molecular biology (Horwitz, M.S., "Adenoviridae and Their Replication," in Virology, 2nd edition, Fields et al., eds., Raven Press, New York, 1990). The viral genes are classified into early (known as E1-E4) and late (known as L1-L5) transcriptional units, referring to the generation of two temporal classes of viral proteins. The demarcation between these events is viral DNA replication. The human adenoviruses are divided into numerous serotypes (approximately 47, numbered accordingly and classified into 6 subgroups: A, B, C, D, E and F), based upon various properties including hemaglutination of red blood cells, oncogenicity, DNA base and protein amino acid compositions and homologies, and antigenic relationships.

Recombinant adenoviral vectors have several advantages for use as gene transfer vectors, including tropism for both dividing and non-dividing cells, minimal pathogenic potential, ability to replicate to high titer for preparation of vector

stocks, and the potential to carry large inserts (Berkner, K.L., Curr. Top. Micro. Immunol. 158:39-66, 1992; Jolly, D., Cancer Gene Therapy 1:51-64, 1994).

The cloning capacity of an adenovirus vector is proportional to the size of the adenovirus genome present in the vector. For example, a cloning capacity of about 8 kb can be created from the deletion of certain regions of the virus genome dispensable for virus growth, *e.g.*, E3, and the deletion of a genomic region such as E1 whose function may be restored in trans from 293 cells (Graham, F.L., J. Gen. Virol. 36:59-72, 1977) or A549 cells (Imler et al., Gene Therapy 3:75-84, 1996). Such E1-deleted vectors are rendered replication-defective. The upper limit of vector DNA capacity for optimal carrying capacity is about 105%-108% of the length of the wild-type genome. Further adenovirus genomic modifications are possible in vector design using cell lines which supply other viral gene products in trans, *e.g.*, complementation of E2a (Zhou et al., J. Virol. 70:7030-7038, 1996), complementation of E4 (Krougliak et al., Hum. Gene Ther. 6:1575-1586, 1995; Wang et al., Gene Ther. 2:775-783, 1995), or complementation of protein IX (Caravokyri et al., J. Virol. 69:6627-6633, 1995; Krougliak et al., Hum. Gene Ther. 6:1575-1586, 1995).

Adenoviral vectors for use in gene transfer to cells and in gene therapy applications commonly are derived from adenoviruses by deletion of the early region 1 (E1) genes (Berkner, K.L., Curr. Top. Micro. Immunol. 158:39-66, 1992). Deletion of E1 genes renders the vector replication defective and significantly reduces expression of the remaining viral genes present within the vector. However, it is believed that the presence of the remaining viral genes in adenovirus vectors can be deleterious to the transfected cell for one or more of the following reasons: (1) stimulation of a cellular immune response directed against expressed viral proteins, (2) cytotoxicity of expressed viral proteins, and (3) replication of the vector genome leading to cell death.

Transgenes that have been expressed to date by adenoviral vectors include p53 (Wills et al., Human Gene Therapy 5:1079-188, 1994); dystrophin (Vincent et al., Nature Genetics 5:130-134, 1993; erythropoietin (Descamps et al., Human Gene Therapy 5:979-985, 1994; ornithine transcarbamylase

(Stratford-Perricaudet et al., Human Gene Therapy 1:241-256, 1990; We et al., J. Biol. Chem. 271;3639-3646, 1996;); adenosine deaminase (Mitani et al., Human Gene Therapy 5:941-948, 1994); interleukin-2 (Haddada et al., Human Gene Therapy 4:703-711, 1993); and α1-antitrypsin (Jaffe et al., Nature Genetics 1:372-378, 1992); thrombopoietin (Ohwada et al., Blood 88:778-784, 1996); and cytosine deaminase (Ohwada et al., Hum. Gene Ther. 7:1567-1576, 1996).

The particular tropism of adenoviruses for cells of the respiratory tract has relevance to the use of adenovirus in gene transfer for cystic fibrosis (CF), which is the most common autosomal recessive disease in Caucasians. Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene that disturb the cAMP-regulated Cl^- channel in airway epithelia result in pulmonary dysfunction (Zabner et al., Nature Genetics 6:75-83, 1994). Adenovirus vectors engineered to carry the CFTR gene have been developed (Rich et al., Human Gene Therapy 4:461-476, 1993) and studies have shown the ability of these vectors to deliver CFTR to nasal epithelia of CF patients (Zabner et al., Cell 75:207-216, 1993), the airway epithelia of cotton rats and primates (Zabner et al., Nature Genetics 6:75-83, 1994), and the respiratory epithelium of CF patients (Crystal et al., Nature Genetics 8:42-51, 1994). Recent studies have shown that administering an adenoviral vector containing a DNA sequence encoding CFTR to airway epithelial cells of CF patients can restore a functioning chloride ion channel in the treated epithelial cells (Zabner et al., J. Clin. Invest. 97:1504-1511, 1996; U.S. Patent No. 5,670,488, issued September 23, 1997).

Transfer of the cystic fibrosis transmembrane conductance regulator (CFTR) cDNA to airway epithelia of patients with cystic fibrosis (CF) thus provides an example of successful use of gene transfer to correct a cellular defect, *i.e.*, the CF defect in electrolyte transport. Vector systems including adenoviral vectors (Zabner et al. (1993) Cell 75: 207; Knowles et al. (1995) New Engl. J. Med. 333: 823; Hay et al. (1995) Hum. Gene. Ther. 6: 1487; Zabner et al. (1996) J. Clin. Invest. 97: 1504 and U.S. Patent No. 5,670,488) and cationic lipids (Caplen et al. (1995) Nat. Med. 1: 39 and U.S. Patent No. 5,650,096) have been shown to be capable of transferring the CFTR cDNA and expressing CFTR in mature ciliated human airway epithelia. The successful delivery of CFTR in such cells is manifest in the appearance of a functional

chloride ion channel in the treated cells.

While CFTR cDNA can be delivered to target cells for expression, current adenoviral vectors are less than optimal in delivering the CFTR cDNA to airway epithelia because the binding of the virus to the apical (exposed) surface of the epithelium is limited. Grubb et al. (1994) Nature 371: 802. The limited infection can be partially overcome by increasing the contact time between the virus and the apical surface. Zabner et al. (1996) J. Virol. 70: 6994.

Cationic lipid vector-mediated gene transfer to mature human airway epithelia is also suboptimal. Caplen et al. (1995) Nat. Med. 1: 39. While it appears that cationic molecules bind to the cell surface and in some cases are taken up by the cell, important barriers to transgene expression may be release of DNA from the endosome, entry into the nucleus, release of DNA from the cationic molecule, and transcription of the DNA. Zabner et al. (1995) J. Biol. Chem. 270: 18997.

Gene transfer systems that combine viral and nonviral components have been reported. Cristiano et al. (1993) Proc. Natl. Acad. Sci. USA 90: 11548; Wu et al. (1994) J. Biol. Chem. 269: 11542; Wagner et al. (1992) Proc. Natl. Acad. Sci. USA 89: 6099; Yoshimura et al. (1993) J. Biol. Chem. 268: 2300; Curiel et al. (1991) Proc. Natl. Acad. Sci USA 88: 8850; Kupfer et al. (1994) Hum. Gene Ther. 5: 1437; and Gottschalk et al. (1994) Gene Ther. 1: 185. In most cases, adenovirus has been incorporated into the gene delivery systems to take advantage of its endosomolytic properties. The reported combinations of viral and nonviral components generally involve either covalent attachment of the adenovirus to a gene delivery complex or co-internalization of unbound adenovirus with cationic lipid: DNA complexes. Further, the transferred gene is contained in plasmid DNA that is exogenous to the adenovirus. In these formulations, large amounts of adenovirus are required, and the increases in gene transfer are often modest.

Accordingly, there is a need in the art for improved vector systems for the efficient delivery of transgenes to target cells. The present invention overcomes certain limitations associated with adenoviral vectors and while retaining the desirable features of the vector system.

SUMMARY OF THE INVENTION

The present invention is an adenovirus complex of a cationic molecule and of an adenovirus having at least one polyalkalene glycol polymer bound thereto. Exanples of polyalkalene glycol polymers that can be used include, but are not limited to, polyethylene glycol, methoxypolyethyleneglycol, polymethyl-ethyleneglycol, polyhydroxypropyleneglycol, polypropylene glycol, and polymethylpropylene glycol, in which polyethylene glycol is more preferred. The polyethylene glycol polymers have an average molecular weight of from 200 daltons to 20,000 daltons, with 2000 daltons to 12,000 daltons being preferred, and about 5000 daltons being even more preferred.

In another embodiment of the present invention, the polyalkalene glycol poymer is an activated polyalkylene glycol polymer. Examples of activated polyalkylene glycol polymer that can be used include, but are not limited to, methoxypolyethylene glycol-tresylate (TMPEG), methoxypolyethylene glycol-acetaldehyde, methoxypolyethylene glycol activated with cyanuric chloride, N-hydroxysuccinimide polyethylene glycol (NHS-PEG), polyethyleneglycol-N-succimimide carbonate and mixtures thereof.

The adenovirus component of the complex is preferably a recombinant adenoviral vector. Adenoviral vectors containing a transgene such as a nucleic acid encoding CFTR are particularly preferred.

In accordance with the present invention, the polyalkalene glycol polymer is directly covalently bound to the virus particle, indirectly covalently bound to the virus particle by an intermediate coupling moiety, directly noncovalently attached to the virus particle, or indirectly noncovalently attached to the virus particle by a ligand. The ligand for indirect noncovalent attachment is preferably a ligand having specificity for a viral surface component, such as an antibody. One particularly preferred antibody to be used is a non-neutralizing anti-adenovirus antibody, such as a non-neutralizing anti-hexon antibody.

The cationic molecule component of the complexes of the present invention is a cationic polymer, with DEAE-Dextran being preferred. Alternatively, the cationic molecule is a cationic lipid.

In another embodiment, the present invention provides a composition containing the above-described adenovirus complexes in a carrier.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows capillary electropherographs of adenovirus treated with 3% (w/v) TMPEG and MPEG.

Fig. 2 is a graph of the time course of mobility change on capillary electropherographs of adenovirus treated with 3 % (w/v) TMPEG.

Figs. 3A-D show photon correlation spectroscopy results demonstrating the change in viral particle size during PEGylation.

Fig. 4 depicts infectivity (CPRG) assay results for a single addition of 3 % TMPEG, 3% MPEG and control virus exposed for 0-6h.

Figs. 5A-E depict infectivity (CPRG) assay results for stepwise additions of 5% PEG_{5000}, PEG_{12000}, or PEG_{20000}.

Figs. 6A-C depict infectivity (chemiluminescence, RLU) assay results for stepwise additions of 3%, 5% or 8% PEG_{5000}.

Figs. 7A-C depict infectivity (chemiluminescence, RLU) assay results for stepwise additions of 5% PEG_{5000}.

Figs. 8A-C depict infectivity (chemiluminescence, RLU) assay results for stepwise additions of 5% PEG_{12000} and PEG_{20000}.

Fig. 9 depicts infectivity (chemiluminescence, RLU) assay results for a single addition of 3 % PEG_{5000}.

Figs. 10A - C show graphs of an antibody neutralization assay for the impact of stepwise additions of 5% PEG_{5000} on neutralization of infectivity (chemiluminescence, RLU assay), 10,000:1 antibody molecules to virus particles.

Figs. 11A - C show graphs of antibody neutralization assays for the impact of stepwise additions of 5% PEG_{5000} on neutralization of infectivity (chemiluminescence RLU assay); 5,000:1 antibody molecules to virus particles.

Figs. 12A - C show graphs of an antibody neutralization assay for the impact of stepwise additions of 5% PEG_{12000} on neutralization of infectivity (chemiluminescence RLU assay); 10,000:1 antibody molecules to virus particles.

Figs. 13A - C show the elution profile of control and TMPEG-treated virus from DEAE ion exchange resin following chromatography.

Fig. 14 depicts comparative infectivity (chemiluminescence, RLU) assay results as illustrated by transgene expression in naive and immunized mice infected with PEGylated or sham treated adenoviral vectors.

Fig. 15 depicts infectivity (chemiluminescence, RLU) assay results in naive mice for (i) adenovirus alone, (ii) adenovirus with 10% $TMPEG_{5000}$, (iii) adenovirus/10% $TMPEG_{5000}$ complexed with poly-L-lysine , and (iv) adenovirus/10% PEG_{5000} complexed with DEAE-Dextran.

Fig 16 depicts comparative infectivity (chemiluminescence, RLU) assay results in naive and immunized mice for (i) adenovirus with 10% $TMPEG_{5000}$, and (ii) adenovirus/10% $TMPEG_{5000}$ complexed with DEAE-Dextran, (iii) sham-treated adenovirus (10% $MPEG_{5000}$) and (iv) sham-treated adenovirus (10% $MPEG_{5000}$) complexed with DEAE-Dextran.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides complexes of cationic molecules and polymer-modified adenovirus that advantageously exhibit increased infectivity and reduced immunogenicity. The cationic complexes of the present invention have surprisingly been found to exhibit heightened levels of infectivity in cells previously immunized with adenovirus, in addition to heightened levels of infectivity in naive (*i.e.*, non-immunized) cells.

In accordance with the present invention, adenoviral vector particles are polymer-modified by covalently or noncovalently binding to the virus a polyalkalene glycol polymer, which renders the viral vector substantially non-immunogenic. The polyaklene glycol polymers used in the present invention preferably have an average molecular weight of from about 200 to about 20,000 daltons. Examples of glycol polymers that can be used include, but are not limited to, polyoxymethylene glycols, polyethylene glycols (PEG), methoxypolyethylene glycols, and derivatives thereof including for example polymethyl-ethylene glycol, polyhydroxypropylene glycol, polypropylene glycol, and polymethylpropylene glycol.

A preferred glycol polymer used in accordance with the present invention is PEG. PEG is a water-soluble polymer having the formula $H(OCH_2CH_2)_nOH$, wherein n is the number of repeating units and determines the average molecular weight. PEGs having average molecular weights of from 200 to 20,000 daltons are commercially available from a variety of sources. In accordance with the present invention, PEG having an average molecular weight of from 200 (PEG_{200}) to 20,000 ($PEG_{20,000}$)can be used to prepare adenoviruses modified with PEG. In a preferred embodiment, PEG has an average molecular weight from about 2000 to about 12,000, with an average molecular weight from about 4000 to about 6000 (*e.g.*, 5000) being more preferred.

In accordance with the invention, the adenoviruses are polymer-modified by direct covalent, indirect covalent, or indirect noncovalent attachment of the polyaklalene glycol polymer to the virus particle. A variety of schemes exist for covalent and non-covalent attachment: 1) the glycol polymer can be attached via direct covalent coupling to the surface of the adenovirus; 2) the glycol polymer can be attached via indirect covalent coupling (*e.g.*, via an intermediate coupling moiety that links the polymer to the adenovirus surface); or 3) the glycol polymer can be attached via an indirect non-covalent linkage using, for example, a suitable PEGylated ligand. Examples of suitable ligands include, but are not limited to, antibodies to surface proteins, lipids or carbohydrates.

Targets for polymer modification include reactive groups on the viral surface with which the polymer or coupling agent can interact, including for example primary and secondary amine groups, thiol groups and aromatic hydroxy groups. As will be apparent to one of ordinary skill in the art, the preferred method for polymer modification of the adenovirus is dependent upon the available target sites found on the viral surface. Examples of available target sites for attachment of the glycol polymer to the adenovirus include, but are not limited to, the hexon, penton cell base, and fiber proteins. The adenoviral hexon protein is a particularly preferred site for attachment of the alkalene glycol polymer. While not wishing to be bound by theory, it is believed that modification of these sites masks epitopes from neutralizing antibodies thereby providing the adenoviral vector with reduced antigenicity and/or

immunogenicity.

Methods for the direct or indirect covalent attachment of polymers to polypeptides that are known in the art may be used to provide the polymer-modified adenoviruses of the present invention. Methods are described, for example, in WO 90/04606, and in U.S. Patent Nos. 4,179,337 and 5,612,460, the disclosures of which are incorporated herein by reference. Generally, the glycol polymer is activated by converting a terminal moiety of the polymer to an activated moiety, or by attaching an activated coupling moiety to the polymer. The activated polymer is then coupled to the target via the activated moiety. The activated moiety or activated coupling moiety can be selected based upon its affinity for the desired target site on the viral surface.

For example, the terminal hydroxyl groups of PEG can be converted into reactive functional group or attached to an activated coupling moiety to provide a molecule known as "activated" PEG. Various forms of activated PEG are known in the art and are commercially available. For direct covalent linkage to the adenovirus a suitable activated PEG is MPEG-tresylate (TMPEG), which is believed to react with ϵ-lysine groups, or MPEG-acetaldehyde. For indirect covalent linkage other forms of activated PEG are known in the art and commercially available, including for example methoxypolyethylene glycol (MPEG) derivatives such as MPEG activated with cyanuric chloride, PEG N-hydroxysuccinimide PEG (NHS-PEG), which reacts with amine groups, and PEG-N-succimimide carbonate. These and other activated PEGs are disclosed in W095/06058, and in U.S. Patent Nos. 4,179,337 and 5,612,460, which are incorporated herein by reference.

The covalent attachment of PEG to the adenovirus surface ("PEGylation") is accomplished by incubating the virus with the activated PEG (*e.g.*, TMPEG). Single addition or multiple addition incubation regimes can be used. The optimal ratios of TMPEG to adenoviral particles to achieve reduced antigenicity, along with heightened infectivity, may be ascertained by performing the various assays described below. Under conditions designed to provide direct TMPEG modified adenovirus, PEGylation in the amount of about 5-20% w/v is preferred, with a concentration of about 10% w/v being most preferred.

Preferably, when high concentrations (*e.g.,* 10% or greater) of glycol polymer are attached to the virus, the activated polymer is added in a stepwise fashion. Stepwise addition is preferred since viral particles tend to aggregate at high concentrations, which reduce the overall effeciency of PEGylation. Moreover, aggregation is exacerbated by the use of certain activated polymers, *e.g.,* TMPEG. Thus, the inital use of low polymer concentrations in a stepwise manner can reduce the tendency of the particles to aggregate, thereby facilitating a higher degree of PEGylation. For example, activated PEG such as TMPEG may be added in separate steps to a viral stock solution every thirty minutes to increase the polymer concentration each time by 3%, 5% or 8% (w/v) in the reaction mixture to obtain final polymer concentrations of 12%, 20% and 32% respectively (approximately w/v, *i.e.*, not correcting for the volume of the polymer). In addition, after the last addition of the glycol polymer, a further incubation time might be allowed. These necessary adjustments to the reaction parameters (*e.g.*, the number of steps, concentration of the polymer, and reaction time) for optimal results can easily be ascertained by one of ordinary skill in the art..

The attachment reaction may be quenched by dialysis or by addition of excess lysine (*e.g.*, a 10 to100-fold excess lysine). Alternatively, the reaction might be run to completion (*i.e.,* the point at which the activated PEG, such as TMPEG, is either completely consumed in the PEGylation reaction or rendered inactive by hydrolysis).

In another embodiment of the present invention, the glycol polymer is indirectly noncovalently attached to the adenovirus via a suitable ligand. In a preferred embodiment, the ligand is an antibody or antibody fragment, including for example a non-neutralizing anti-virus antibody or fragment therefrom (*e.g.*, Fab, $F(ab')_2$, Fv). As used herein, the term "antibody" includes monoclonal and polyclonal antibodies. In a particularly preferred embodiment, the ligand is a non-neutralizing anti-hexon antibody. Such antibodies are commercially available and include, for example, MAb 8052 and MAb 805 available from Chemicon International, Temecula, CA, USA.

Indirect non-covalent attachment of glycol polymer to the adenovirus is accomplished by incubation of the virus with a suitable ligand that has been modified by the covalent attachment of polymer. The glycol polymer can be covalently attached (*i.e.*, bound) to the ligand by standard methods as described herein above. For example, a non-neutralizing anti-virus antibody such as anti-hexon antibody may be PEGylated using an activated PEG molecule as described above. In a preferred embodiment, anti-hexon antibody is modified using TMPEG. One of ordinarly skilled in the art can ascertain the optimal ratios of activated PEG to antibody, concentrations of activated PEG and antibody, buffer and time and temperature of incubation to achieve optimal modification of the antibody. The polymer-modified ligand is then incubated with adenovirus to allow non-covalent binding of the polymer-modified ligand to the virus surface.

Antibodies modified with PEG at the epitope binding site (*e.g.*, complementarity determining regions (CDRs)) can exhibit reduced affinity to the adenovirus thereby decreasing the efficiency noncovalentl attachment. In order to prevent PEGylation at the epitope binding site an antibody is preferably immobilized prior to PEG modification. For example, anti-hexon antibody is bound to purified immobilized hexon (*e.g.*, hexon-Sepharose®) prior to PEG modification of antibody. The PEGylated antibody is then released from immobilized hexon.

Alternately, non-immobilizied anti-hexon antibodies can be PEGylated creating a population of antibodies PEGylated on the epitope binding site in addition to other sites, which are thereafter separated by immunoaffinity chromatography. For example, the mixed population of modified antibodies can be incubated with immobilized hexon, to which antibodies modified only at sites other than the epitope binding site will bind. These PEGylated antibodies are then released from the immobilized hexon for use in accordance with the present invention.

For some applications, for example, those requiring repeat dosing of a polymer modified virus, it may be desirable to separate the unreacted glycol polymer from the polymer-modified adenovirus, which may then be purified by standard methods as necessary for the intended use. Separation and purification may be performed by methods known in the art, for example ion exchange chromatography,

gel filtration chromatography, or cesium chloride gradient purification. In situations in which there is indirect PEGylation of an antibody, hexon affinity resin may be useful to separate the PEGylated antibody from unreacted PEG.

In addition, it may be desirable to separate unmodified adenovirus from polymer-modified adenovirus. Separation of the unmodified from polymer-modified virus may be performed by partitioning in an aqueous biphasic polyalkylene glycol solution. For example, phase partitioning in an aqueous biphasic system of PEG and dextran may allow the separation of PEG-modified virus from unmodified virus. Partitioning may be performed by counter-current distribution. Generally, the phase system is prepared by mixing solutions of dextran and PEG. PEG and PEG-modified virus are incorporated into the phase system, mixed by inversion or rotation, and allowed to separate. PEG modified virus partitions into the PEG phase, and unmodified virus partitions into the dextran phase.

The efficiency of adenovirus polymer modification (*e.g.*, PEGylation) is evaluated by methods known in the art, including ion exchange chromatography, capillary electrophoresis (CE), photon correlation spectroscopy (PCS), and through the use of a labeled (*e.g.*, biotinylated) PEG in a quantitative ELISA. Ion exchange chromatography (*e.g.*, DEAE-chromatography) can be performed by standard methods to evaluate the modified viruses based upon altered charge.

Whole virus CE provides a means to monitor the modification of adenovirus by the glycol polymer as a function of altered surface charge. For example, covalent attachment of PEG to the adenovirus surface seems to result in shrouding of the negative surface charges on the viral particle thereby causing virus to exhibit a more neutral mobility. CE may be performed by methods known to those of ordinary skill in the art. For instance, a ramped low-high voltage pre-treatment is used to electrophorese the highly mobile salt ions in which the virus may be formulated for stability, before true, high voltage separation begins. In plots derived from CE, virus particles with PEG covalently attached run at a position closer to the neutral point than virus without covalently attached PEG. CE may be conveniently used to assess the influence of various conditions, including molar ratios, concentrations and incubation times, on the covalent attachment of PEG to the virus particles. Increasing

neutrality reflects increasing PEG-chain density on the virus surface.

PCS uses the relationship between particle size and movement in suspension (via Brownian motion) to gain accurate measurements on the size of the particles. This method is widely applied to monitor polymer attachment to particles including liposomes, microspheres and nanoparticles by measuring their increase in size. These data suggest that covalently attached PEG at relatively low density forms globular "mushroom" shapes and thus the increase in size is relatively small. Altering the conditions under which one would expect to increase the density of covalently attached PEG chains results in a more extended conformation of the polymer or "brush" shapes which is reflected by a relatively larger increase in particle size. Thus PCS may be used using methods known to those of ordinary skill in the art to monitor the size changes of the virus particle under different reaction conditions.

The ELISA analysis of a biotinylated PEG can provide the most quantitative assessment of the number of molecules of PEG covalently bound to the adenovirus virus particle. The ELISA can be performed by standard methods known in the art.

The indirect noncovalent attachment of glycol polymer via a polymer-modified ligand can also be monitored by displacement of labeled ligand from the adenovirus in a competition enzyme-linked immunosorbent assay (ELISA). For example, the ability of a PEGylated anti-hexon antibody to bind to the adenovirus surface is measured in a standard competition ELISA using a biotinylated anti-hexon antibody.

As used herein, the term "adenovirus" includes genetically engineered adenoviruses (*i.e.*, recombinant adenoviral vectors). Preferably, the adenovirus of the present invention is a recombinant adenovirus engineered to be incapable of replicating and exhibits minimal expression of adenoviral genes. Suitable recombinant adenovirus include adenoviral vectors derived from adenovirus type 2 (Ad2), type 5 (Ad5) and type 6 (Ad6) which have been deleted for the E1 regions. Representative adenoviral vectors that are useful for delivery of a transgene are disclosed by Zabner et al. (1996) J. Clin. Invest. 6 : 1504, Zabner et al. (1993); Cell 75 : 207, U.S. Patent Nos. 5,707,618 and 5,670,488, the disclosures of which are

incorporated herein by reference.

The recombinant adenoviruses also preferably contain transgenes operably linked to suitable promoter and other regulatory sequences. "Transgenes" are defined herein as nucleic acids that are not native to the adenovirus. Examples of transgenes to be utilized are nucleic acids encoding a biologically functional protein or peptide, an antisense molecule, or a marker molecule. The promoter may be an endogenous adenovirus promoter, for example the E1a promoter or the Ad2 major late promoter (MLP) or a heterologous eucaryotic promoter, for example a phosphoglycerate kinase (PGK) promoter or a cytomegalovirus (CMV) promoter. Similarly, those of ordinary skill in the art can construct adenoviral vectors utilizing endogenous or heterologous poly A addition signals.

In a preferred embodiment, the recombinant adenoviral vector contains a transgene such as the nucleic acid encoding cystic fibrosis transmembrane conductance regulator (CFTR). CFTR is a phosphorylation and nucleoside triphosphate-regulated Cl^- channel located in the apical membrane of epithelial cells in the lung, intestine, pancreas and sweat glands. For a review, see Welsh et al., (1992) Neuron 8: 821, incorporated herein by reference. Cystic fibrosis (CF) results from a non-functional Cl^- channel in an individual's epithelial cells caused by mutations in the gene encoding CFTR. Such mutations result in loss of function of the chloride channel and thus defective electrolyte transport in affected epithelial cells. DNA encoding wild-type CFTR is known in the art; the sequence is disclosed, for example, in U.S. Patent No. 5,670,488, incorporated herein by reference. A deletion mutant of CFTR that encodes a regulated Cl^- channel is disclosed by Sheppard et al. (1994) Cell 76: 1091, and in , U.S. Patent Nos. 5,670,488 and 5,639,661, the disclosures of which are incorporated herein by reference. Other examples of recombinant adenoviral vectors containing transgenes encoding CFTR are Ad2/CFTR-2, Ad2/CFTR-8, and Ad2/CFTR-16, which are respectively found in Zabner et al. (1996) J. Clin. Invest. 97(6):1504-1511, U.S. Patent No. 5,707,618 and U.S. Serial No.: 08/839,552 filed April 14, 1997, all incorporated herein by reference.

In accordance with the present invention, DNA encoding a CFTR protein includes the foregoing published sequences as well as other DNAs encoding

CFTR known to those of skill in the art. Further included are modifications of the known DNA molecules, for example mutations, substitutions, deletions, insertions and homologs, that encode a functional CFTR protein, *i.e.*, a chloride channel.

In another embodiment of the invention, the polymer-modified recombinant adenovirus is an adenovirus that can induce tumor-specific cytolysis also known as viral oncolysis. Representative adenovirus that are useful for viral oncolysis are disclosed by Bischoff et al. (1996) Science 274:373; Heise et al. (1997) Nature Medicine 3:630; and EP689447A, the disclosures of which are incorporated herein by reference.

In accordance with the present invention, the polymer-modified adenovirus is complexed with a cationic molecule. The cationic molecule can be any cationic compound that exhibits minimal toxicity to mammals and does not decrease the infectivity of the polymer-modified virus. Preferably, the cationic molecule provides the polymer-modified virus with infectivity levels comparable to, if not greater than, the infectivity levels exhibited by the corresponding unmodified adenovirus. Examples of cationic molecule that can be used include, but are not limited to, cationic polymers, cationic lipids, cationic sugars, cationic proteins, or cationic dendrimers. The cationic molecules can also be combined with non-cationic molecules. Examples of cationic polymers include, but are not limited to, polyethyleneimine (PEI), DEAE-dextran, and histone (fraction V-S), protamine, polybrene (Hexadimethrine Bromide) and cationic dendrimers, in which DEAE-dextran is preferred. Alternatively, the polymer-modified adenovirus can be dispersed in a metal salt precipitate such as calcium phosphate, as set forth in pending application U.S. Serial No. 09/082,510, filed May 21, 1998, which is incorporated herein by reference. Those of ordinary skill in the art can determine the molecular weight of the cationic polymer that provides optimal gene transfer in accordance with the methods described herein. PEI is preferably used at an average molecular weight of 25 kDa.

Cationic lipids are known to those of ordinary skill in the art. Representative cationic lipids include those disclosed *e.g.*, by U.S. Patent No. 5,283,185, PCT/US95/16174 (WO96/18372) and U.S. Patent No. 5,650,096, the

disclosures of which are incorporated herein by reference. In a preferred embodiment the cationic lipid is (N-(N[1],N[1]-dimethylaminoethane) carbamoyl] cholesterol (DC-Chol) disclosed in U.S. Patent No. 5,283,165. In another preferred embodiment, the cationic lipid is N^4-spermine cholesterol carbamate (GL-67) or N^4-spermidine cholesterol carbamate (GL-53) disclosed in WO96/18372 and U.S. Patent No. 5,650,096. Other representative cationic lipids include (2, 3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA), dioctadecylamidoglycyl spermine (DOGS), commercially available as TRANSFECTAM® from Promega, Madison, WI; 1,3-dioleoyloxy-2-(6-carboxyspermyl)-propyl amide (DOSPER); N-[1-(2,3-Dioleoyloxy)propyl] -N,N,N-trimethyl-ammoniummethylsulfate (DOTAP); N-[1-2(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA); (±)-N-(2-Hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide (DMRIE); (±)-N-(2-Aminoethyl)-N,N-dimethyl-2,3-bis (tetradecyloxy)-1- propanaminium bromide (βAE-DMRIE); dimethyldioctadecylammonium bromide (DDAB); LIPOFECTIN®, a 1:1 (w/w) formulation of DOTMA and dioleoyl phosphotidylethanolamine (DOPE) commercially available from Life Technolgies, Gaithersburg, MD; LIPOFECTAMINE®, a 3:1 (w/w) formulation of DOSPA and DOPE commercially available from Life Technologies, Gaithersburg, MD; LIPOFECTACE™, a 1:2.5 (w/w) formulation of DDAB and DOPE, commercially available from Life Technologies, Gaithersburg, MD; Tfx™ -50, a reagent consisting of N,N,N', N'-tetramethyl-N-N'-bis(2-hydroxyethyl)-2,3,-dioleoyloxy-1, 4-butanediammonium iodide and DOPE, commercially available from Promega, Madison, WI; and DMRIE-C™, a 1:1 (molar ratio) formulation of DMRIE and cholesterol commercially available from Life Technologies, Gaithersburg, MD. In a preferred embodiment the cationic lipid is GL-53 or GL-67. In accordance with the present invention, the cationic lipid may be combined with a colipid such as DOPE or cholesterol.

The ratio of cationic molecule to polymer-modified adenovirus to be used in complex formation is variable. As will be apparent to one skilled in the art, factors that may affect the cationic molecule : virus ratios include the cationic molecule selected, polyalkylene glycol polymer selected, and cell type targeted for

infection. However, optimal cationic molecule : virus ratios can easily be determined by one skilled in the art utilizing the infectivity assays described herein. Some illustrative cationic molecule : virus ratios for the complexes of the present invention are set forth below. DEAE-Dextran is complexed with the polymer-modified adenovirus at a ratio of 100-3000 molecules per virus particle, with 400-600 molecules per virus particle being preferred. PEI is complexed at a ratio of 200-1200 molecules per virus particle, with 400-600 molecules per virus particle being preferred. Protamine is complexed with 400-40,000 molecules per virus particle, with 3000-5000 molecules per virus particle being preferred. Polybrene is complexed with 4×10^3 - 4×10^5 molecules per virus particle, with 3.5×10^5 - 4.5×10^5 molecules per virus particle being preferred. The cationic lipid, GL-67, is complexed with 9×10^5 - 9×10^6 molecules per virus particle, with 8.5×10^6 - 9.5×10^6 molecules per virus particle being preferred. Alternatively, calcium phosphate can be co-precipitated in the presence of the polymer-modified virus by admixing a molar excess of calcium (Ca^{2+}) to phosphate (PO_4^-), (Ca^{2+} : PO_4^-), ranging from 6:1 to 42:1, with a ratio 13:1 to 15:1 being preferred.

The complexes of the present invention can be simply prepared by admixing the components under suitable conditions. For example, suspensions of viral particles and cationic molecules are prepared with phosphate-buffered saline (PBS) at pH of 7. Prior to admixing the two suspension, the suspensions are warmed to 30°C to facilitate complex formation. The two suspensions are mixed and incubated at 30°C for approximately 15 minutes to allow sufficient complex formation. However, if desired, complex formation can be conducted at room temperature with additional incubation times. The complexed polymer-modified adenovirus is then resuspended in PBS and is ready for administration to a host.

As previously described, the cationic complexes of polymer-modified (*e.g.*, PEGylated) adenovirus exhibit heightened levels of infectivity in both immune subjects and naive (*i.e.*, non-immune) subjects. However, PEGylation of the virus exceeding 15% can cause a decrease or ablation of viral infectivity, thereby providing a disincentive for further PEGylation. Normally, decreases in viral infectivity do not occur at PEGylation levels of 10% or less depending on the glycol polymer selected

for attachment (*e.g.*, TMPEG vs. MPEG). Accordingly, the cationic complexes of the present invention provide a solution to this problem by allowing significantly greater levels of polymer modification to be used (*e.g.*, 20% or greater) while maintaining viral infectivity levels comparable to unmodified (*i.e.*, non-polymer-modified) adenovirus. In fact, as demonstrated in the examples set forth below, the complexes of the present invention provide infectivity levels significantly greater that either unmodified adenovirus and polymer-modified adenovirus.

Infectivity of the adenoviral complexes of present invention are assessed by standard infection assays. For example, the ability of adenovirus to infect a cell is assessed by monitoring the expression of a transgene (*e.g.*, a reporter gene such as lacZ) contained within the adenovirus. Genetic reporter systems are well-known in the art, and are disclosed for example in Short Protocols in Molecular Biology, 1995, Ausubel et al., eds., 3rd edition, Wiley and Sons, Inc. The adenoviral vector is engineered by standard methods to contain a transgene, and the complexed adenoviral vector is used to infect cells that are permissive for the virus. After infection under standard conditions, cell lysates are analyzed for the presence of the product of the transgene, *e.g.*, β-galactosidase. For example, the product of the transgene can be assessed by colorimetric, chemiluminescence or fluorescence assays, or immunoassays. In this way, those of ordinary skill in the art can compare complexed and uncomplexed adenoviral vector, and can determine the optimal percentages and conditions for glycolization and cationic complexing that result in optimum retention of infectivity.

Alternatively, if the transgene encodes CFTR, infectivity can be measured by acsertaining the ability of the CFTR protein expressed in cultured CF airway epithelia to correct the Cl^- channel defect following the methods described by Rich et al. (1990) Nature 347: 358, incorporated herein by reference. Briefly, cultured CF airway epithelial cells are infected with adenoviral vectors containing DNA encoding a CFTR protein. Virus-mediated expression of functional CFTR protein is assessed using an SPQ [6-methoxy-N-(3-sulfopropyl)-quinolinium, Molecular Probes, Eugene, OR] halide efflux assay. SPQ is a halide-sensitive fluorophore, the fluorescence of which is quenched by halides. In this assay, cells are loaded with

SPQ, CFTR is activated by cAMP agonists, the CFTR Cl^- channel opens, halides exit the cell, and SPQ fluorescence in the cell increases rapidly. Thus increases in intracellular fluorescence in response to cAMP provide a measure of a functional Cl^- channel.

In another assay suitable for measuring viral infectivity, CF epithelial cells are infected with adenoviral vectors containing DNA encoding a CFTR protein, and secretion of Cl^- from infected cells is measured in response to cAMP stimulation. The secretion of Cl^- can be measured as an increase in transepithelial short-circuit current with addition of cAMP agonists, as described for example by Rich et al. (1993) Human Gene Therapy 4: 461, the disclosure of which is incorporated herein by reference. Expression of a functional CFTR protein can also be assessed by patch clamp techniques that detect reversibly activated whole-cell currents in response to addition of cAMP agonists, or single-channel currents in excised, cell-free patches of membrane in response to cAMP-dependent protein kinase and ATP. Patch clamp techniques are described for example by Sheppard et al. (1994) Cell 76: 1091, and U.S. Patent No. 5,639,661, the disclosures of which are incorporated herein by reference.

Retention of infectivity is defined herein as an infectivity level sufficient to have therapeutic value, for example at least about 20% infective relative to unmodified virus (non-complexed, non-polymer-modified adenovirus). For some embodiments, the virus complex maintains at least 60% infectivity. In other therapeutic embodiments, the complexed modified virus is preferred to maintain at least 80% infectivity. Lower percent infectivity of at least 5% may be useful for applications such as viral oncolysis.

In a particular example of an infectivity assay, an adenoviral vector containing the β-galactosidase (β-gal) reporter gene (lacZ) is covalently modified by exposure to various concentrations of TMPEG and subsequently complexed with a cationic molecule, other than poly-L-lysine (*e.g.*, DEAE-dextran). A cell line that supports adenoviral vector propagation, for example 293 human embryonic kidney cells (ATCC CRC 1573), is exposed to unmodified and modified/complexed adenoviral vector containing the β-gal gene. Cells are then incubated under

conditions appropriate for β-gal expression. The presence of β-gal in cell lysates is measured by standard colorimetric, fluorescence, or chemiluminescence assays, *e.g.*, by using X-gal. The quantity of β-gal in 293 cell lysates provides a measurement of the ability of the complexed, PEGylated adenovirus to infect 293 cells. The complexed, PEGylated virus that maintains 50% infectivity relative to unmodified virus is considered to retain infectivity.

The complexed, polymer-modified adenoviruses of the present invention exhibit reduced antigenicity relative to unmodified virus. Reduced antigenicity is defined as a statistically significant ($p>0.05$) reduction in binding of the polymer-modified virus to neutralizing antibodies against the virus. Reduced antigenicity is assessed by methods known in the art, including *in vitro* and *in vivo* assays. For example, both modified and unmodified viruses containing reporter genes are incubated in the presence or absence of neutralizing antibodies or serum. The antibody-treated viruses and non-antibody treated control viruses are then used to infect cells as described above, and reporter gene expression in infected cells is performed as described above. With unmodified viruses, treatment with neutralizing antibodies results in lower levels of infection and thus lower levels of transgene expression. The complexed, polymer-modified adenoviruses of the present invention are protected from neutralization by the polymer coating, and thus provide increased infectivity and increased transgene expression in the present assays relative to unmodified viruses that have been exposed to neutralizing antibodies.

By utilizing the foregoing assays, those of ordinary skill in the art can determine the conditions for glycolization and subsequent complexing necessary to provide a complexed, polymer-modified adenovirus that maintains infectivity and exhibits reduced antigenicity.

Because of their unique properties, the cationic complexes of polymer-modified adenoviruses are particularly useful for therapeutic and diagnostic <u>in vivo</u> applications. The cationic complexes of the present invention have utility in medical therapy and diagnosis in medical and veterinary practice and in agriculture. They are of particular use in gene therapy (for example the delivery of genes for the localized expression of a desired gene product) and for non-gene therapy applications such as,

but without limitation, viral oncolysis. The viruses are useful, for example, to deliver genes, toxins and/or diagnostic markers. An additional application is in the creation of tolerogens for viral antigens.

In one embodiment of the present invention, a method is provided for introducing a transgene into a target cell. The method comprises introducing into the target cell the complexed, polymer-modified adenovirus of the present invention, wherein the adenovirus is a recombinant adenoviral vector comprising the transgene. The complexed, polymer-modified adenoviruses are particularly useful for delivering a transgene to a target cell for the treatment of various disorders, for example in which the transgene product is absent, insufficient, or nonfunctional. Alternatively, the expression of the transgene may serve to block the expression or function of an undesired gene or gene product in the target cell.

Target cells for adenovirus complexes of the present invention are any cell in which expression of a transgene is desired. Target cells include cell types permissive to adenovirus infection (*e.g.*, 293 cells and A549 cells) and cell types resistant to adenovirus infection (*e.g.*, human epithelial cells, NIH 3TC cells, and 9L gliosarcoma cells). In fact, the complexed, polymer-modified adenoviral vectors are particularly suitable for infecting adenovirus resistant cells for transgene expression. While not wishing to be bound by theory, the complexes of the present invention do not require binding to the Coxsackie Adenovirus Receptor (CAR) for internalization. As will apparent to the skilled artisan, internalization of adenovirus in permissive cell types is generally dependent on the CAR pathway. However, the complexes of the present are internalized by pathways other than CAR, which renders them particularly suitable for transgene expression in adenovirus resistant cell types (Fasbender et al., (1997) J. Biol. Chem. 272:6479-6489; Kaplan et al., (1998) Hum. Gen. Ther. 9:1469-1479).

The complexed, polymer-modified adenovirus is introduced into the host cell by methods known in the art, including for example infection. Infection of a target cell in vivo is accomplished by contacting the target cell with the adenovirus. The adenovirus is delivered as a composition in combination with a physiologically acceptable carrier. As used herein, the term "physiologically acceptable carrier"

includes any and all solvents, diluents, isotonic agents, and the like. In a preferred embodiment the complexed, polymer-modified adenovirus is a recombinant adenoviral vector polymer-modified by covalent attachment of PEG and subsequently complex with DEAE-dextran. The use of such media and agents for compositions is well known in the art. The adenoviruses of the invention may be delivered to the target cell by methods appropriate for the target cell, including for example by ingestion, injection, aerosol, inhalation, and the like. The compositions may be delivered intravenously, by injection into tissue, such a brain or tumor, or by injection into a body cavity such as pleura or peritoneum.

The formulation of compositions is generally known in the art and reference can conveniently be made to Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Co., Easton, PA. The forms of the present complexes suitable for administration include sterile aqueous solutions and dispersions. The subject complexed, polymer-modified adenoviruses are compounded for convenient and effective administration in effective amounts with a suitable physiologically acceptable carrier and/or diluent.

The effective amounts of the complexed, polymer-modified adenovirus to be used in accordance with the present invention for humans, or any other mammal, can be determined by the ordinary skilled artisan with consideration of individual differences in age, weight and condition of the subject.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated, each unit containing a predetermined quantity of active material calculated to produce the desired effect in association with the required carrier. The specification for the novel dosage unit forms of the invention are dictated by and directly depend on the unique characteristics of the polymer-modified viruses and the limitations inherent in the art of compounding. In the case of compositions containing supplementary active ingredients, the dosages are determined by reference to the usual dose and manner of administration of the ingredients.

The present invention further provides a method for delivering a virus to a tumor, comprising administering a complexed, polymer-modified adenovirus of the invention to a subject in need of such treatment under conditions whereby the adenovirus localizes to a tumor. The ability of the complexed adenoviruses of the present invention to provide retention of infectivity and reduced impact of neutralizing antibodies open up this additional method of use for polymer-modified virus. Particulates of the size range 100-200nm undergo passive tumor targeting in relation to the so-called EPR effect (Enhanced Permeability and Retention). Tumors have leaky vasculature and thus long circulating particles have the opportunity to leave the circulation and enter the tumor parenchyma via the holes in tumor blood vessels. Tumors lack lymphatics which is the main system for removal of macromolecules and particles from the tissues (the basis for the Retention element in EPR). PEG has been used to enhance the passive targeting of liposomes to tumors via increased circulation time. However, data in the scientific literature shows that this approach leads to unfavorable properties such as unacceptable low tumor to blood ratios (*i.e.* less than 1) for much of the lifetime of the product. Using different optimization principles it has been shown (WO 96/34598) that additional effects of PEGylation, other than improved circulation time, can be exploited to solve this problem and achieve both good tumor localization and high tumor to blood ratios as well as high tumor to normal tissue ratios. Thus the present invention provides a means of improving the tumor localization of virus particles. This is relevant to both gene therapy applications where viral vectors are used to deliver genes and for non-gene therapy applications. The latter include the recently discovered system selective for the infection of p53 deficient tumor cells which has the capacity to kill tumor cells via viral oncolysis Bischoff JR, Kirn DH, Williams A, Heise C, Horn S, Muna M, Ng L, Nye JA, Sampson-Johannes A, Fattaey, McCormick F (1996) Science 274:373-376; Heise et al. (1997) Nature Medicine 3:369-645; and EP689447A, incorporated herein by reference.

The invention is further illustrated by the following specific examples which are not intended in any way to limit the scope of the invention.

EXAMPLES

Example 1

Covalent Attachment of Polyethylene Glycol to Adenovirus

Tresyl-monomethoxypolyethylene glycol (TMPEG) was prepared using $MPEG_{5000}$. In this example and in subsequent examples, except where otherwise indicated, TMPEG was prepared as set out in WO 95/06058, which corresponds to U.S. Application Serial Nos. 08/471,348 and 08/601,040, filed June 6, 1995 and February 23, 1996, respectively, the disclosures of which are incorporated herein by reference.

Type 2 adenovirus (genetically modified to carry the β-gal reporter gene), as disclosed in U.S. Patent No. 5,670,488, was prepared by banding in isopycnic CsCl density centrifugation (three rounds), then extensively dialysed against phosphate buffered saline (PBS, pH 7.2) containing 5% sucrose. The stock solution used contained 6.4×10^{10} infectious units per ml (4.8×10^{11} particles/ml). The virus stock was made 3%w/v by the addition of dry TMPEG, typically 3.0mg to 100μl of stock. The samples were incubated at 25°C with rotary mixing for 24h.

The polymer-treated virus was monitored via capillary electrophoresis (CE) using a Beckman P/ACE 5010 system with a 57cm silica capillary of 50μm Internal diameter (inlet=anode). A preliminary 1.5min wash in 1M NaOH and second wash in running buffer (20mM phosphate buffer pH 7.0, 5.0mM NaCl) were performed. After incubation, the samples were transferred to the CE machine where the auto sampler removed a few nanolitres by a pressure injection setting of 10s and separation was achieved using 2 minute voltage ramping to a final of 17Kv.

Whole virus CE monitors the changes in surface charge of the virus upon treatment with PEG. Incubation with PEG correlates with a progressively increased more neutral mobility to the virus. Increasing neutrality is consistent with an increased PEG-chain density on the virus surface.

Figure 1 (upper panel) shows superimposed capillary electropherographs for adenovirus exposed to 3%(w/v) TMPEG and MPEG. The hiatus in each plot marks the trough at the point of neutrality. The TMPEG treated

virus ran at a location significantly nearer the neutral point than the sham-treated MPEG. Under these PEGylation conditions there is no evidence of residual unPEGylated virus (*i.e.* no peak or shoulder on the TMPEG trace corresponding to the control virus).

In order to confirm that the mobility shift was not an artifact, a mixture of equal volumes of the two samples was loaded (Figure 1, lower panel). Two well separated peaks were evident, corresponding to those shown in the upper panel.

Figure 2 shows the time course of the change in electrophoretic mobility of virus with duration of exposure to TMPEG 3% (w/v), prepared essentially as described, above using 300μl of virus stock and 3%(w/v) TMPEG. The % mobility was calculated as follows: (mobility of modified virus peak- mobility of neutral position)/(mobility of unmodified virus peak-mobility of neutral position) X 100. Since the reaction co-product can influence the running buffer, this was renewed at the point arrowed: 100μl of reaction mixture was analyzed up to this point (using the repeat sampling function of the CE machine, *i.e.* without mixing) and a fresh 100μl aliquot of the reaction mixture was used thereafter.

Example 2

Covalent Attachment of Polyethylene Glycol to Adenovirus

Type 2 adenovirus stock solution prepared as in Example 1 (1.35×10^{10} infectious units per ml; 9.3×10^{11} particles per ml) was PEGylated using 3%(w/v) TMPEG except that rotary mixing was not used so that repeated size analyses could be made.

Viral particle size was monitored using photon correlation spectroscopy (PCS) in a Malvern Instrument's ZetaMaster 5.

Figures 3A and 3B show the diameter versus time for TMPEG treated and untreated virus respectively. Results are expressed as % time 0 values. Figures 3C and 3D show measurements taken during a PEGylation reaction over a longer time period. Reaction with TMPEG is shown in Figure 3D and sham treatment with MPEG is shown in Figure 3C. Treatment with TMPEG results in an increase in particle size (Figs. 3B and 3D) which is not seen in the control untreated virus (Fig.

3A) or in the MPEG treated virus (Fig. 3C). Increases in size are shown in Figs. 3B and 3D. PCS has the advantage of giving numeric data and thus the method gives an ability to rank samples.

Example 3

Infectivity Assays for PEGylated and Sham Treated Virus

Several regimes of PEG treatment were evaluated with respect to retention of infectivity (see also Example 4). In addition to exposure to 3%(w/v) TMPEG, stepwise addition was also used (the objective being to achieve higher ultimate PEGylation). The rationale behind step wise addition is that viral particles tend to aggregate and this is exacerbated by PEG, especially at high concentrations. However, PEGylation has been shown, in the context of other particles (*e.g.* liposomes), to prevent aggregation. Thus initial PEGylation at low polymer concentration can serve to reduce the tendency to aggregate at subsequent higher polymer concentrations and hence achieve a higher degree of PEGylation. Three step wise addition regimes were used: TMPEG or MPEG were added every thirty min to viral stock solution (prepared as in Example 1) to increase the polymer concentration by 3%, 5% or 8% in the reaction mixture. Viral stocks used for these experiments ranged from 1.35-7.6x10 infectious units per ml and $9.3\text{-}20 \times 10^{11}$ particles per ml. In each experiment a maximum of four additions of dry polymer were made, equating to final polymer concentrations of 12 %, 20 % and 32% (~w/v, *i.e.* not correcting for the volume of the polymer). In some experiments the 4th addition was sampled after 30 mins and a further incubation time (giving 5 reaction conditions).

Infectivity was measured in two ways (see also Example 4). β-gal expression was monitored in human 293 cells (Graham et al., J. Gen. Virol. 36:59-72, 1977) exposed to virus in culture (this cell line is permissive for adenoviral replication). Cells were trypsinised 1 day prior to assay and seeded at 400μl per well in a 24 well microliter plate using a 1×10^6/ml cell suspension. Having established a monolayer by 24h, 10μl of reaction mixture was added to each of 4 replicate wells containing 293 cells. The cells were incubated overnight in a fully humidified atmosphere of 5% CO_2 in air at 37°C to express β-gal.

The cell monolayer was depleted of medium and then washed with PBS. Then 60μl of lysis buffer (15 % triton X-100, 25OmM Tris-HCl, pH 7.0) was added and the microliter plate incubated at room temperature for 30 min in an orbital shaker. After the cells had lysed for 30 min 50μl of each sample was transferred to a fresh microliter plate. A set of β-gal standards (5.5 units in lysis buffer and doubling dilutions in lysis buffer) was added to the same microliter plate. 150 μl of CPRG substrate buffer (1.6mM CPRG, 60mM phosphate buffer: 1mM $MgSO_4$; 10mM KC1; 50mM β-mercaptoethanol; 250 ml distilled water) was added to each well. After brief mixing (4 min) the plate was read at 555 nm on a microliter plate reader (Titertek Multiskan Plus MKII, ICN, flow Laboratories, Switzerland).

The single addition of 3 % (w/v) TMPEG was examined using the CPRG assay. Figure 4 shows the results of CPRG assays on TMPEG treated virus (open circles) and MPEG sham-treated virus (triangles) and control virus (filled circles). None of the treatments produced a trend of falling infectivity over the time period studied (six hours). A second independent experiment confirmed this result, showing no significant decline in OD over 6 hours for either control virus, TMPEG treated virus or virus sham-treated with MPEG (data not shown). Thus the PEG treatment of virus in Examples 1 and 2 demonstrated no reduction in infectivity.

The stepwise addition of 5% of PEG_{5000}, PEG_{12000} or PEG_{20000} produced a variable impact on infectivity (Figure 5). Panels A and B show the impact of stepwise addition of 5% of PEG_{5000} (mean of 2 and mean ±SD of 4 replicates respectively, some error bars are hidden by the symbols). The TMPEG (filled circles) produced a reduction in infectivity as compared to the MPEG (open circles). With PEG_{12000} (panels C and D, same symbols), in one experiment TMPEG decreased the infectivity of the virus as compared with the MPEG treated virus, but in the other, MPEG and TMPEG were not significantly different (*i.e.* MPEG and TMPEG had a similar effect on infectivity). Treatment with $TMPEG_{20000}$ also did not show any significantly greater effect than the equivalent amount of $MPEG_{20000}$ (Panel E same symbols).

Example 4

Infectivity Assays for PEGylated and SHAM Treated Virus

Single and stepwise additions of TMPEG and MPEG were prepared as in Example 3 and analyzed with respect to infectivity using a chemiluminesent reporter assay system for the detection of the virally encoded β-galactosidase (Galacto-Light™). This assay system uses a chemiluminescent substrate and was performed in accordance with the manufacturer's instructions.

Figures 6A - C compares the effects of 3%, 5% and 8% incremental additions of $TMPEG_{5000}$ (filled circles) or $MPEG_{5000}$ (open circles) on viral infectivity. Note that in Figure 6A and B the MPEG and TMPEG treated viral samples show similar infectivity. A modest decline in infectivity with treatment with either MPEG or TMPEG was observed. In subsequent experiments with no-PEG controls these showed a similar decline in infectivity, suggesting that this was a handling effect and not due to PEG. In Figure 6C the MPEG and the TMPEG treated virus performed similarly. Thus, this experiment shows that treatment with TMPEG or MPEG does not result in loss of infectivity.

Apparent loss of infectivity due to the addition of PEG chains was seen twice with this assay in experiments using PEG_{5000} in the 5% incremental addition scheme (Figures 7A and B, filled circles TMPEG- open circles MPEG). A subsequent assay of the same sample as shown in Figure 7B showed no significant difference between the MPEG and TMPEG treatments, indicating that no significant loss of infectivity had in fact occurred (Figure 7C, same symbols).

Figure 8 shows comparable results for PEG_{12000} (panels A and B, filled circles TMPEG; open circles MPEG) and PEG_{20000} (panel C, same symbols). As above, condition 0 is an untreated virus control and conditions 1-4 are stepwise additions of 5 % TMPEG or MPEG. With the PEG_{12000} there was a modest additional loss of infectivity with TMPEG in one of the two experiments after the 3rd and 4th addition of TMPEG (panel B). In the other experiment (Panel A) using PEG_{12000} no significant reduction in infectivity was observed with either TMPEG or MPEG. With PEG_{20000}, TMPEG treatment produced lower infectivity than MPEG for all additions including the first, but approximately one third the initial infectivity value remained

even after the 4th addition of TMPEG.

With a single addition of 3% PEG_{5000}, *i.e.* prepared as in Example 3, with the chemiluminescence assay there was a modest decline in infectivity (Figure 9). It should be noted that the decline in infectivity observed over time was seen both in the case of the TMPEG (filled circles) and MPEG treated virus (open circles) as well as the untreated "handling" control (triangles).

Example 5

The Impact of PEGylation on the Reduction of Infectivity by Neutralizing Antibodies

Using the infectivity assay given in Example 4, exposure of the TMPEG and MPEG treated virus to neutralizing antibodies was used to seek evidence of the protection from neutralization afford by the polymer treatment.

Transgene expression was monitored in the presence and absence of a polyclonal neutralizing antibody purified from rabbit anti-hexon serum using a hexon affinity resin. The polyclonal antibody was titered with untreated virus and the ratio was established where 30 to 50% infectivity was retained in the presence of the neutralizing antibody. Two antibody titers were used 10,000:1 (~30%) or 5,000:1 (~40-50%) (antibody molecules to virus particles) where indicated.

Figures 10-12 show the impact of incremental additions of 5% $TMPEG_{5000}$ (Figures 10 and 11) and $TMPEG_{12000}$ (Figure 12) to the adenovirus on antibody neutralisation (B Panels) compared to incremental addition of MPEG (A Panels) to the virus. The open circles in the A and B panels of Figures 10 - 12 are infectivity in the absence of antibody; the closed circles are infectivity in the presence of antibody.

In all three cases there is evidence of significant protection from neutralization and a trend of improving protection with the highest/longest TMPEG exposure giving maximum protection. The A and B panels in each figure show the raw data, while Figures 10C, 11C, and 12C show the transgene expression as a percent of the equivalent non-antibody treated control. In each of the C panels the open bars indicate transgene expression from MPEG treated virus while the hatched bars indicate transgene expression from TMPEG virus. In Figure 10 the amount of

virus added to the assay was adjusted to compensate for differences in the number of infectious units of the non-antibody treated controls. In Figures 11 and 12 the same number of viral particles was assayed for each condition. The antibody titers were 10,000:1, 5,000:1 and 10,000:1 respectively.

These data show protection from immune recognition. For the purposes of clarification, protection is defined as there being a statistically significant difference in transgene expression in the presence of the immune agent under test (*e.g.* antibody or cell suspension) as compared with the expression observed in untreated control.

The single addition of 3% $TMPEG_{5000}$ showed some protection after 4h and 6h incubation in two independent assays. Taken in conjunction the above examples indicate the presence of a PEGylation "window" where treatment with PEG does not abrogate all infectivity but conveys statistically significant protection from neutralisation by antibody.

Example 6

Quantitative Analysis OFPEGylated Adenoviral Vector

An Ad2/β-gal 2 vector (U.S. Patent No. 5,670,488 and described by Zabner et al. (1996) J. Virol. 70 : 6994) was covalently modified by PEG with 0.01%, 0.1%, 1.0% or 5.0% biotinylated NHS-PEG_{5000} (Shearwater Polymers). PEGylated vector proteins were analyzed by SDS-PAGE. SDS-PAGE demonstrated that the hexon, penton base and fiber were the primary targets for covalent modification by PEG, and increasing concentration of PEG led to modification of additional proteins.

PEGylation of adenovirus was also assessed quantitatively. Ad 2-β-gal 2 vector was treated with increasing amounts of TMPEG-biotin 5%, 10%, or NHS-PEG-biotin 0.01%, 0.1%, 1%, 5%. Both PEG_{5000}'s were obtained from Shearwater Polymers. Stepwise additions of PEG were made every 30 minutes up to a period of 1 hour for TMPEG-biotin and 2 hours for NHS-PEG-biotin. Following PEG treatment the unreacted PEG was separated from the PEG-virus by CsCl gradient purification and the amount of PEG-biotin attached to the virus was quantitated using an ELISA assay with an avidin HRP conjugate as reporter. A standard curve of PEG-biotin (0-

250 ng/ml) was generated to determine the number of molecules of PEG-biotin attached per virus particle. Results are shown in Table 1.

Table 1

Sample	Molecules PEG-biotin:virus particle
0.1% NHS-PEG-Biotin	600:1
1% NHS-PEG-Biotin	3077:1
5% NHS-PEG-Biotin	3191:1
5% TMPEG-Biotin	1500:1
10% TMPEG-Biotin	1000:1

Treatment of adenovirus with either TMPEG-biotin or NHS-MPEG-biotin led to the covalent attachment of PEG-biotin to the surface of the virus. The data indicates that at comparable concentrations of tresyl and NHS PEG-biotin, more PEG-biotin was attached to the virus particle after treatment with the NHS-PEG biotin, which is consistent with reports that the reaction of NHS-PEG with lysine residues occurs more quickly (30-45 minutes) compared to the reaction of tresyl MPEG with lysine residues which occurs over an extended period of time (2-3 hours).

This data provides quantitative results regarding the extent of covalently bound PEG.

Example 7

Covalent Attachment of Polyethylene Glycol to Adenovirus

Type 2 adenovirus (genetically modified to carry the β-gal reporter gene) was prepared by banding with isopycnic CsCl density centrifugation then extensively dialysed against phosphate buffered saline (PBS pH 7.2). Three different types of MPEGs were tested for their ability to PEGylate adenovirus namely a) cyanuric chloride activated $MPEG_{5000}$ b) $TMPEG_{5000}$ and c) amino-PEG_{5000}. The MPEGs were obtained from Shearwater Polymers. Activation of MPEG with cyanuric chloride couples one triazine ring per MPEG molecule. This activated

MPEG can react with amino groups on proteins. Alternatively MPEG can be activated with tresyl chloride (2,2,2,-trifluoroethanesulphonyl chloride) to form tresylated MPEG which can react with epsilon amino groups on proteins to form a highly stable amine linkage. SPDP-amino MPEG couples to proteins via cysteine residues. The activated NHS ester end of SPDP reacts with the amine groups on the amino PEG to form an amide linkage. The 2-pyridyldithiol group at the other end is free to react with sulfhydryl groups to form a disulfide linkage. SPDP - aminoPEG was synthesized by the addition of SPDP (N-succinimidyl 3-(2-pyridylditthio) propionate) to amino PEG in the presence of methanol. Following an overnight incubation at room temperature the SPDP-aminoPEG was collected by precipitation with ether.

Ad2-β-gal 2 virus was incubated with either a) cyanuric chloride activated MPEG b) TMPEG or c) amino PEG at increasing ratios of PEG:lysine. Ad2-β-gal 2 virus was dialysed into 0.1M sodium carbonate buffer pH 8.5 containing 0.15M NaCl before treatment with cyanuric chloride activated MPEG or 0.2M sodium phosphate buffer pH 7.5 containing 0.15M NaCl before treatment with TMPEG. All PEGylation reactions were performed at room temperature. Samples were mixed on a rotary platform, the PEGylation reaction was terminated by the addition of excess lysine or alternatively by lowering the temperature. Infectivity of the PEGylated viruses was initially assessed qualitatively by infecting 293 cells with PEGylated virus followed by measurement of transgene expression (β-galactosidase) using X-gal staining. Using this assay the TMPEG treated virus had greater infectivity than the virus that had been treated with cyanuric chloride activated PEG or SPDP-PEG. The TMPEG treated virus was further measured for infectivity using the more quantitative assay of end-point dilution in 293 cells using fluorescence isothiocyanate (FITC)-conjugated anti-hexon antibody as described by Rich, DP, Couture LA, Cardoza LM, Guiggio, VM, Armentano, D., Espino, PC, Hehir, K., Welsh, MJ, Smith, AE and Gregory, RJ, 1993, Hum. Gen. Ther. 4:461-476.

The results are shown in Table 2 and demonstrate that infectivity of the virus is retained following PEGylation with TMPEG. (Error in the assay is ±0.5 log.)

Table 2

PEG:Lysine	Infectivity
5:1	3.8e8 iu/ml
2.5:1	1.5e8 iu/ml
1:1	2.2e8 iu/ml
Control	5e8 iu/ml

Example 8

Reduced Binding of Neutralizing Antibodies to PEGylated Vector

Ad2-β-gal 2 virus was PEGylated with TMPEG as described in Example 11. Virus was incubated with serial two-fold dilutions of neutralizing human serum for 1 h/37°C and 293 cells were added. The assay was read when 293 cells incubated alone reached confluency. The neutralizing titer was defined as the reciprocal of the highest dilution of serum that showed detectable protection of 293 cells from cytopathic effect when compared to cells incubated with virus not exposed to serum. Prior to the assay, the different virus preparations to be tested were titrated to ascertain the lowest dilution that caused 100% cytopathic effect. Results are shown in Table 3.

Table 3

Virus PEG:lysine ratios	Neutralizing titre
5:1	800
2.5:1	3200
Control	6400

According to the results, more serum is required to neutralize the PEGylated virus compared to the untreated virus suggesting that PEGylation covers sites recognized by neutralizing antibodies.

Example 9

Ion-exchange Chromatography of PEGylated Virus Particles

Ad 2-β-gal virus was PEGylated as described in Example 11 with TMPEG at ratios of 50 moles and 10 moles PEG:lysine. The virus was applied to a DEAE ion-exchange resin (Millipore, Bedford, MA) in phosphate buffer containing NaCl. Bound virus was eluted from the resin using an increasing salt gradient and the flow through peaks and eluted protein peaks were analyzed for control virus, virus treated with TMPEG at a ratio of 50:1 PEG:lysine and virus treated with PEG at a ratio of 10:1 PEG:lysine. All samples had equivalent protein values before chromatography.

Figure 13, panel A shows the elution profile from the DEAE-ion exchange resin (Millipore, Bedford, MA) following chromatography of control virus. One main protein peak was eluted from the resin and this was shown to contain infectious virus particles (data not shown). Figure 13, panel B shows the elution profile from the DEAE-ion exchange resin following chromatography of virus that had been treated with TMPEG (10:1 ratio). In contrast to the profile for the control virus there is the appearance of a flow through peak in addition to the eluted protein peak, which has diminished in size. The appearance of the flow through peak suggests that PEGylation has generated viral particles which no longer can bind to the DEAE-resin under these conditions and as a result are now present in the flow through peak along with unreacted PEG. Since ion-exchange chromatography is based on charge interactions between the protein and the ion-exchange resin, apparently PEGylation has produced a heterogenous population of virus particles which have altered surface charges. Those with significant surface charge differences can no longer bind to the resin and are recovered in the flow through peak. The elution profile from the DEAE-ion exchange resin following chromatography of virus PEGylated with TMPEG at a ratio of 50:1 showed a similar profile. The flow through peak in this sample was significantly larger while the eluted protein peak was in contrast reduced. At the increased ratio of PEG:lysine of 50:1 which resulted in a greater fraction of particles eluting in the flow through peak, the virus particles had increased levels of PEGylation. Table 4 expresses the size of the two peaks

(expressed as area under peak) in relation to the PEG:lysine ratios used during PEGylation. In conclusion, ion exchange chromatography may be used to resolve heterogeneous populations of PEGylated virus particles and may be used to separate highly PEGylated virus particles from lightly PEGylated particles on the basis of charge differences.

Table 4

	Flow Through Peak Area	Eluted Peak Area
Control	NA	0.272
PEG-Virus 10:1	0.022	0.132
PEG-Virus 50:1	0.063	0.031

Example 10

Transgene Expression by PEGylated Ad2/β-Gal2 in Immune Mice

Two batches of Type 2 adenovirus stock solution prepared as in Example 1 were mixed (2ml of a batch at 5.38×10^{10} infectious units per ml, 2.055×10^{12} particles per ml and 4 ml of a batch at 1.35×10^{10} infectious units per ml, 9.3×10^{11} particles per ml) and subjected to treatment with PEG using a stepwise addition regime of 5% TMPEG as in Example 3. Samples obtained following two and three additions of TMPEG (*i.e.*, total 10% and 15% TMPEG, respectively) were purified from unreacted TMPEG by a standard CsCl (Sigma Chemical, St. Louis, MO) centrifugation procedure involving a step gradient and two sequential equilibrium gradients. The purified PEG treated vectors were then dialyzed against phosphate buffered saline containing 5% sucrose and frozen at -80°C in small aliquots. The titers were determined by end point dilution on 293 cells using fluorescence isothiocyanate (FITC)-conjugated anti-hexon antibody as described by Rich, DP, Couture LA, Cardoza LM, Giuggio VM, Armentano D, Espino PC, Hehir K, Welsh MJ, Amith AE and Gregory RJ, 1993, Hum. Gen Ther. 4:461-476. The

purified PEG treated viral suspension prepared with total 10% TMPEG contained $2.7x10^{11}$ particles/ml ($3x10^9$ infectious units/ml) and the purified PEG treated viral suspension prepared with total 15% TMPEG contained $2.4x10^{11}$ particles/ml ($6.4x10^8$ infectious units/ml).

The two PEGylated viral suspensions were compared to untreated Type 2 adenovirus ($3.19x10^{10}$ infectious units per ml) for ability to effect gene transfer <u>in vivo</u> in naive and pre-immunized BALB/c mice. Mice were pre-immunized by the intra-nasal administration of 10^9 infectious units of a replication defective Type 2 adenovirus encoding human CFTR (Ad2/CFTR). The animals chosen for the study had serum anti-adenovirus antibody titers of circa 1/25,000 to 1/50,000. Naive BALB/c mice were simply mice that had not been exposed to adenovirus vector. On day 0, the viral preparations were administered as follows: a) untreated virus, $2x10^8$ infectious units were instilled in a volume of 100 μl to each of four mice in the naive group and four mice in the pre-immunized group, b) "PEGylated virus 10%", $3x10^8$ infectious units ($2.7x10^{10}$ particles) were instilled in a volume of 100 μl to each of four mice in the naive group and four mice in the pre-immunized group, c) "PEGylated virus 15%", $6.4x10^7$ infectious units ($2.4x10^{10}$ particles) were instilled in a volume of 100 μl to each of four mice in the naive group and four mice in the pre-immunized group. All animals in the pre-immunized group were subjected to eyebleed on the day of instillation and the blood was analyzed for antibody titers. All mice were sacrificed three days after instillation and lung tissue, right caudal lobe and left lobe, was excised. The right caudal lobe from all four naive and four immunized animals per condition (untreated, "PEGylated virus 10%" and "PEGylated virus 15%") was used for quantification of ß-gal in an AMPGD assay (Galacto-Light™ Kit, Tropix, Bedford, MA). The protein concentration of lung homogenates was determined using the BioRad DC reagent (BioRad, Hercules, CA). The left lobe from two naive and two immunized animals per condition was used for x-gal staining.

Table 5 shows the beta-galactosidase expression per microgram of protein (relative light units, RLU per microgram of protein) for untreated virus, "PEGylated virus 10%" and "PEGylated virus 15%" in both naive and pre-immunized mice. Beta-galactosidase expression in the naive mice was observed for all three viral

preparations in all four mice per condition. In the pre-immunized mice, the untreated vector gives only background levels of beta-galactosidase expression in all four mice. In contrast, the two PEGylated viral preparations gave levels of beta-galactosidase above those for the control animals in 4/4 and 3/4 animals for the "PEGylated virus 10%" and "PEGylated virus 15%" preparations, respectively (see Table 1). Thus PEGylation of the virus conveys protection from neutralization in vivo resulting in substantial expression of the vector in the target tissue in vivo.

Table 5. Beta-Galactosidase expression in lung tissue expressed as relative light units per microgram of protein (RLU/μg protein).

Preparation (infectious units)	Mouse Number	RLU/μg protein Native	RLU/μg protein Immunized
Control virus ($2x10^8$ iu)	1	955	25
	2	1457	90
	3	649	28
	4	1388	38
PEGylated 10% ($3x10^8$ iu)	1	2341	218
	2	2108	1296
	3	3694	164
	4	1730	1964
PEGylated 15% ($6.4x10^7$ iu)	1	705	34
	2	172	305
	3	715	198
	4	1128	108

Example 11

Transgene Expression of PEGylated Ad2/β-gal 4 virus in Immune Mice

Ad2/β-gal 4 virus (U.S. Patent No. 5,670,488) was PEGylated with 10% tresyl MPEG (TMPEG) as already described. PEGylated virus was purified from unreacted TMPEG by banding on cesium chloride gradients (Rich et al., Human Gene Therapy 4:461-476, 1993). The purified PEGylated virus was dialysed into phosphate

buffered saline (PBS), 5% sucrose and the titre was determined by end point dilution on HEK293 cells using fluorescent isothiocyanate (FITC)-conjugated anti-hexon antibody (Rich et al., 1993). Control or sham treated vector was treated with non-reactive MPEG and was purified and titred as described for TMPEG virus. PEGylated and sham treated virus were instilled into immune and naive mice. The dose for each vector was 2×10^8 iu/mouse (equivalent to $\sim 2 \times 10^{10}$ particles), the dose volume per mouse was 100 μl. Immune mice had previously been instilled with Ad2 - CFTR-8 vector (U.S. Patent No. 5,707,618) and had titres to adenovirus in the range 25,000 - 51,200.

Three days after instillation the animals were sacrificed and lung tissue from individual animals were homogenised and β-galactosidase activity in the homogenate was assessed using a commercially available assay kit according to manufacturer's instructions (Galactolight™ Kit, Tropix, Bedfor, MA.). The protein concentration of lung homogenates was determined using the BioRad DC reagent (BioRad, Hercules, CA) and the results expressed as relative light units (RLU)/ug protein.

Figure 14 shows the β-galactosidase expression for PEGylated virus (Ad TMPEG) and sham treated virus (Ad MPEG). Results shown are the mean ± standard deviation of the values obtained with individual animals. β-Galactosidase expression was measured in the lungs of naive mice for both the MPEG and the TMPEG (N=2) viral preparations. In the pre-immunised mice (N=4) the sham treated virus (Ad MPEG) had reduced levels of β-galactosidase expression (~47% of the β-galactosidase expression measured in naive animals), presumably due to neutralisation by adenovirus specific antibodies. In contrast in the pre-immunised mice (N=3) the PEGylated virus gave levels of β-galactosidase expression equivalent to those measured in naive animals (~89% of the expression measured in naive animals). Thus PEGylation of the adenovirus protects the virus from neutralisation, allowing full expression of the vector in the target tissue in the presence of an immune response.

Example 12

Transgene Expression of Cationic Complexes of PEGylated Ad2/β-gal 2 virus in Naive and Immunized Mice

Following the procedure of Example 1, Ad2-β-gal 2 virus was PEGylated with 10% TMPEG (Shearwater Polymers, Huntsville, AL). The PEGylated virus was purified from unreacted TMPEG by banding on CsCl gradients and purified by dialysis against phosphate buffered saline (PBS) as previously described. The titre was determined by end point dilution on 293 cells using fluorescence isothiocyanate (FITC)-conjugated anti-hexon antibody as set forth in Rich et al., (1993) Hum.Gen.Ther. 4:461-476. The PEGylated virus had a titre of 7.6 x 10^8 iu/ml and a particle:iu ratio of 800. Interestingly, the titre of the virus before PEGylation was 9.5 x 10^9 iu/ml with a particle:iu ratio of 80. Thus, viral infectivity was compromised during PEGylation.

Naive mice were instilled with samples of unmodified virus (control), the PEGylated virus and the PEGylated virus complexed with either DEAE-dextran or Poly-L-lysine (PLL). The dose for the control was 2 x 10^8 iu/animal, which was equivalent to 1.0 x 10^{10} particles/mouse ratio. The dose for PEGylated virus (complexed and non-complexed) was 7.6 x 10^7 iu/animal, which was equivalent to 6.4 x 10^{10} particles/mouse. PEGylated virus was complexed with DEAE-dextran at a ratio of 3000 molecules DEAE-dextran per virus particle while the virus was complexed with PLL at a ratio of 500 molecules per virus particle. The dose volume per mouse was 100 ul.

Three days after instillation the animals were sacrificed and lung tissue from individual animals were homogenised and β-gal activity in the homogenate was assessed using the Galactolight™ Kit (Tropix, Bedford, MA) according to manufacturer's instructions. The protein concentration of lung homogenates was determined using the BioRad DC reagent (BioRad, Hercules, CA) and the results expressed as relative light units (RLU)/ug protein. The results are shown in Figure 15.

Figure 15 shows that the PEGylated virus complexed with DEAE-dextran had increased infectivity as compared to the non-complexed PEGylated virus, the unmodified control virus and the PEGylated virus complexed with PLL. Results

shown are the mean ± standard deviation of the values obtained with individual animals.

The infectivity of the above-described adenovirus PEGylated with either TMPEG or MPEG and complexed with DEAE-dextran was also ascertained in naive and immune mice following the procedure set forth above, but with TMPEG from a different supplier being used (Sigma Chemical, St. Louis, MO). The immune mice had previously been instilled with Ad2-CFTR-8 vector and had titres of adenovirus in the range 25000 - 51200. The results are shown in Figure 16.

Figure 16 shows that the PEGylated virus when complexed with DEAE-dextran and administered to naive and immune mice exhibited increased infectivity. Moreover, transgene expression in the immune mice that received virus PEGylated with TMPEG and complexed with DEAE-dextran was equal to that measured for naive mice which demonstrates the reduced antigenicity of the PEGylated virus complexed with DEAE-dextran. In comparison, the transgene expression of virus PEGylated with MPEG and complexed with DEAE-dextran was increased only in naive mice. Thus, virus PEGylated with TMPEG and complexed with DEAE-dextran exhibited both increased infectivity and reduced antigenicity.

CLAIMS

1. An adenovirus complex comprising a complex of a cationic molecule and of an adenovirus having at least one polyalkalene glycol polymer bound thereto.

2. The adenovirus complex of Claim 1, wherein `said polyalkalene glycol polymer is polyethylene glycol.

3. The adenovirus complex of Claim 1, wherein said polyalkalene glycol polymer is methoxypolyethyleneglycol.

4. The adenovirus complex of Claim 1, wherein said polymer is selected from the group consisting of polymethyl-ethyleneglycol, polyhydroxypropyleneglycol, polypropylene glycol, and polymethylpropylene glycol.

5. The adenovirus complex of Claim 2, wherein said polyethylene glycol has an average molecular weight of from 200 daltons to 20,000 daltons.

6. The adenovirus complex of Claim 5, wherein said polyethylene glycol has an average molecular weight of from 2000 daltons to 12,000 daltons.

7. The adenovirus complex of Claim 6, wherein said polyethylene glycol has an average molecular weight of about 5000 daltons.

8. The adenovirus complex of Claim 1, wherein said adenovirus is a recombinant adenoviral vector.

9. The adenovirus complex of Claim 8, wherein said virus is a recombinant viral vector comprising a transgene.

10. The adenovirus complex of Claim 1, wherein said polyalkalene glycol polymer is directly covalently bound to said virus particle.

11. The adenovirus complex of Claim 1, wherein said polyalkalene glycol polymer is indirectly covalently bound to said virus particle by an intermediate coupling moiety.

12. The adenovirus complex of Claim 1, wherein said polyalkalene glycol polymer is indirectly noncovalently attached to said adenovirus particle.

13. The adenovirus complex of Claim 12, wherein said polyalkalene glycol polymer is indirectly noncovalently attached to said virus particle by a ligand.

14. The adenovirus complex of Claim 13, wherein said ligand has specificity for a viral surface component.

15. The adenovirus complex of Claim 14, wherein said ligand is an antibody.

16. The adenovirus complex of Claim 15, wherein said antibody is a non-neutralizing anti-adenovirus antibody.

17. The adenovirus complex of Claim 16, wherein said non-neutralizing anti-virus antibody is a non-neutralizing anti-hexon antibody.

18. The adenovirus complex of Claim 1, wherein said adenovirus is bound to an activated polyalkylene glycol polymer selected from the group consisting of methoxypolyethylene glycol-tresylate (TMPEG), methoxypolyethylene glycol-acetaldehyde, methoxypolyethylene glycol activated with cyanuric chloride, N-hydroxysuccinimide polyethylene glycol (NHS-PEG), polyethyleneglycol-N-succimimide carbonate and mixture thereof.

19. The adenovirus complex of Claim 1, wherein said cationic molecule is a cationic polymer.

20. The adenovirus complex of Claim 19, wherein said cationic polymer is DEAE-Dextran.

21. The adenovirus complex of Claim 1, wherein said cationic molecule is a cationic lipid.

22. A composition comprising the adenovirus complex of Claim 1 and a carrier.

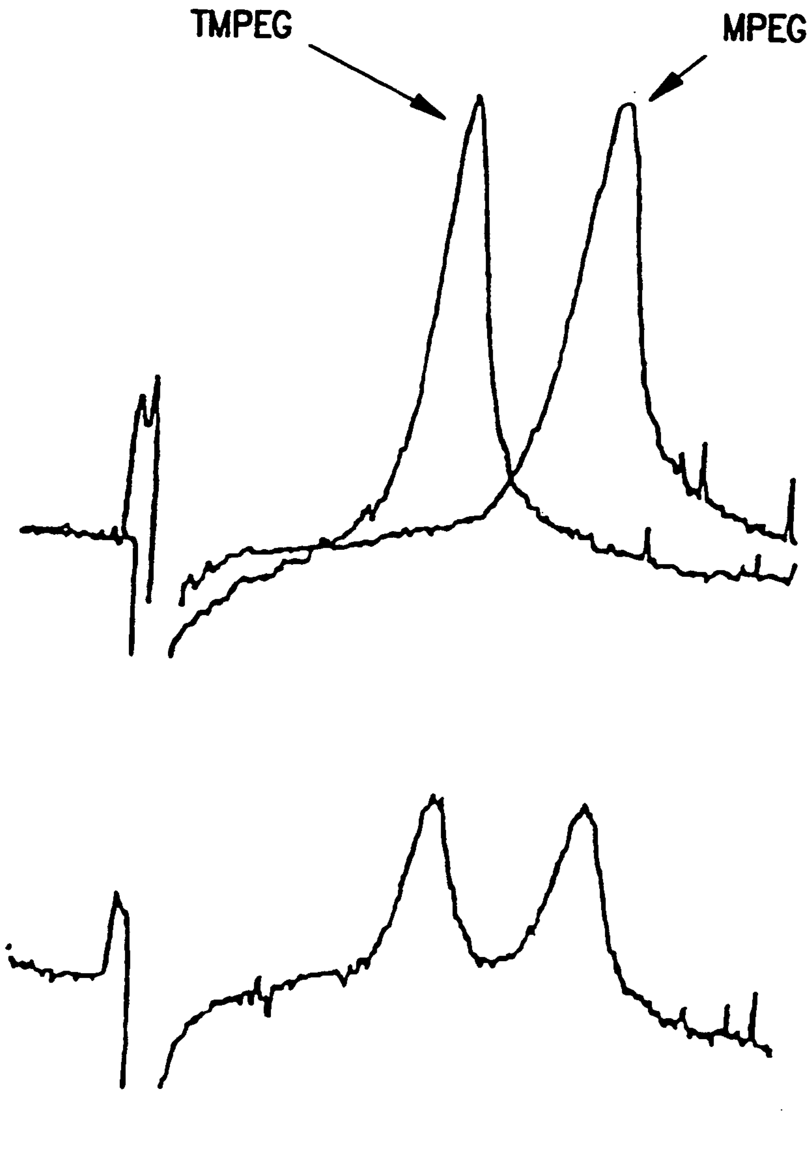


FIG.1

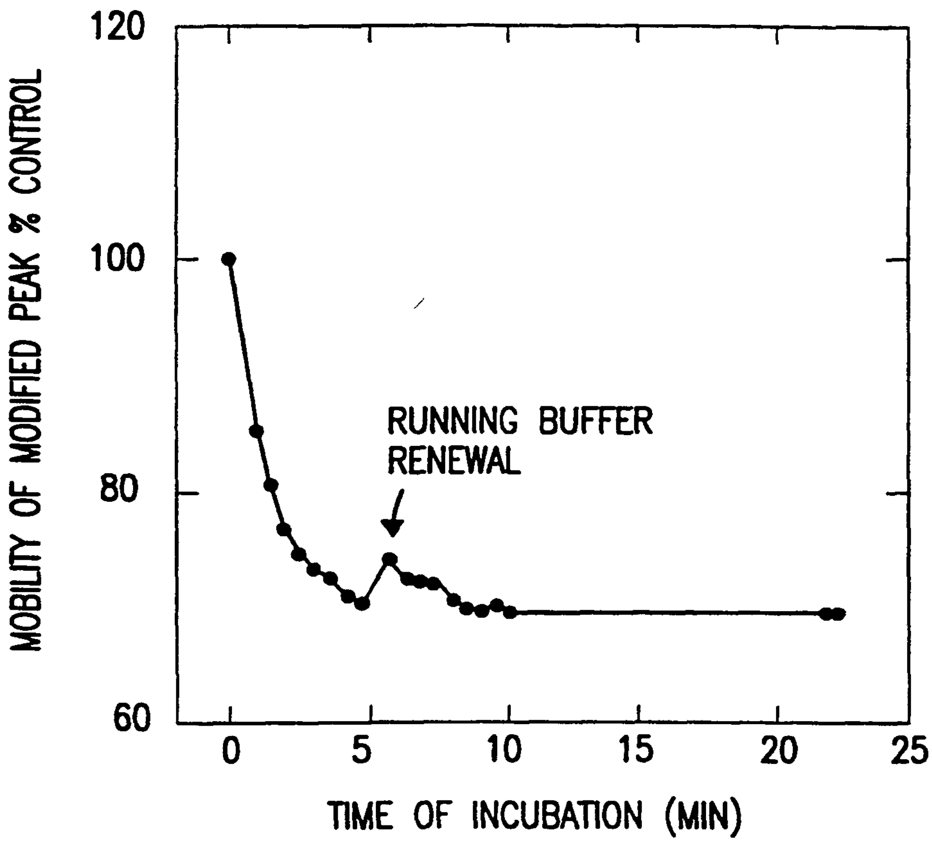

120
100
80
60
MOBILITY OF MODIFIED PEAK % CONTROL
RUNNING BUFFER RENEWAL
0
5
10
15
20
25
TIME OF INCUBATION (MIN)

FIG.2

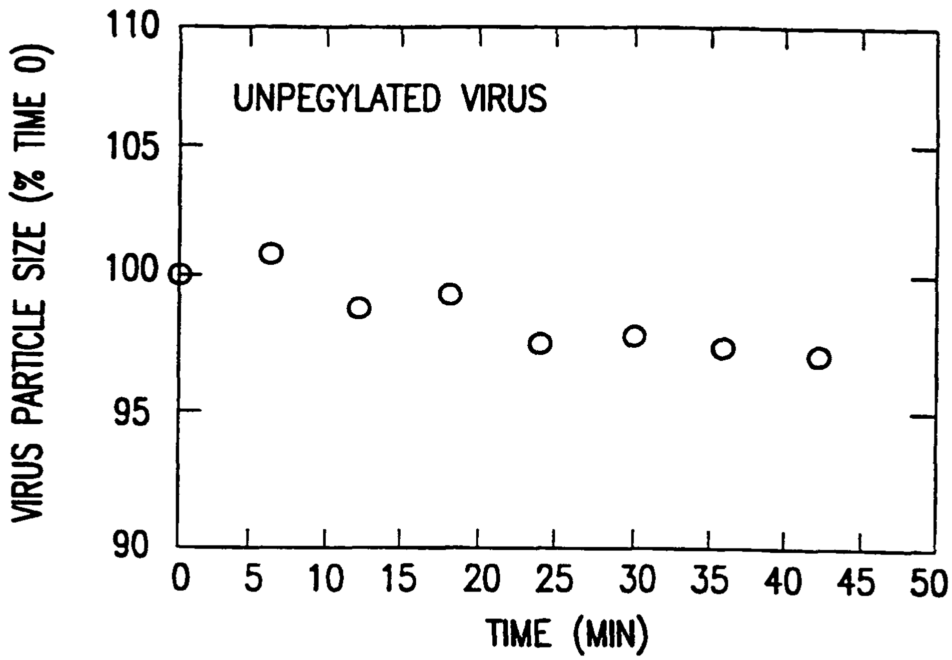

UNPEGYLATED VIRUS
VIRUS PARTICLE SIZE (% TIME 0)
110
105
100
95
90
0
5
10
15
20
25
30
35
40
45
50
TIME (MIN)

FIG.3A

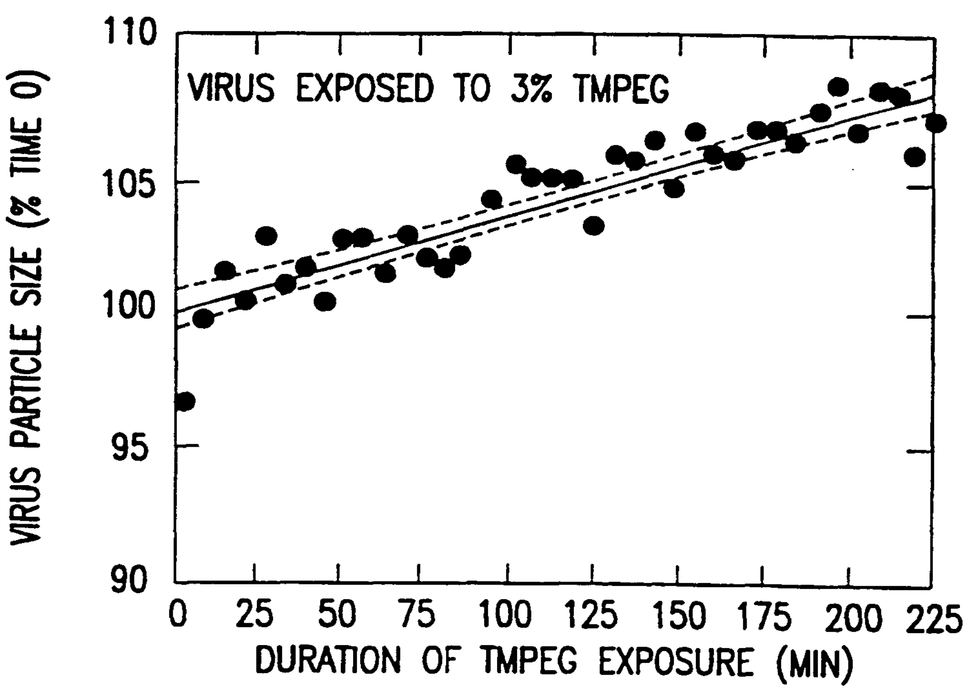

VIRUS EXPOSED TO 3% TMPEG
VIRUS PARTICLE SIZE (% TIME 0)
110
105
100
95
90
0
25
50
75
100
125
150
175
200
225
DURATION OF TMPEG EXPOSURE (MIN)

FIG.3B

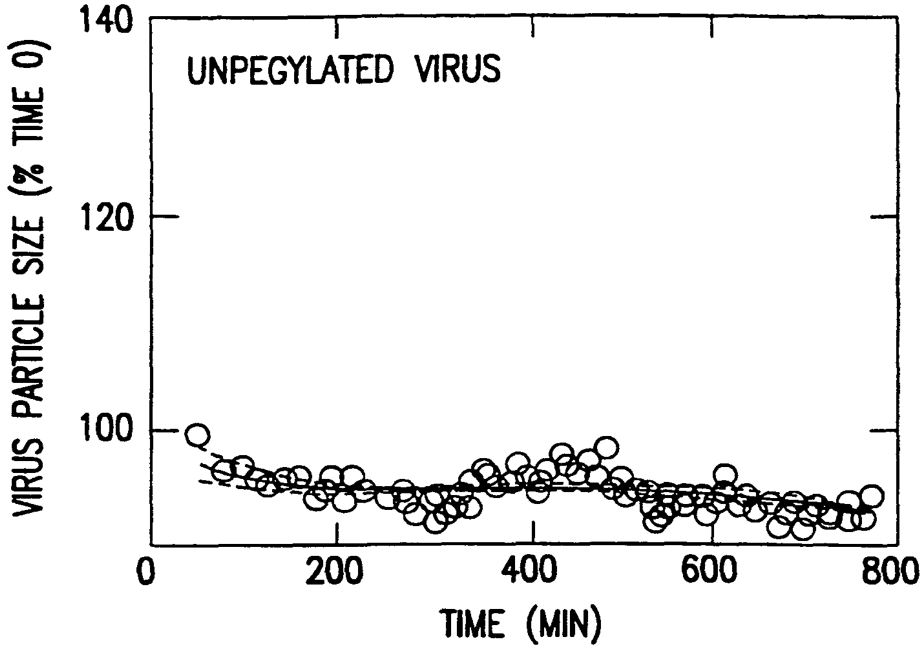

UNPEGYLATED VIRUS
VIRUS PARTICLE SIZE (% TIME 0)
140
120
100
0
200
400
600
800
TIME (MIN)

FIG.3C

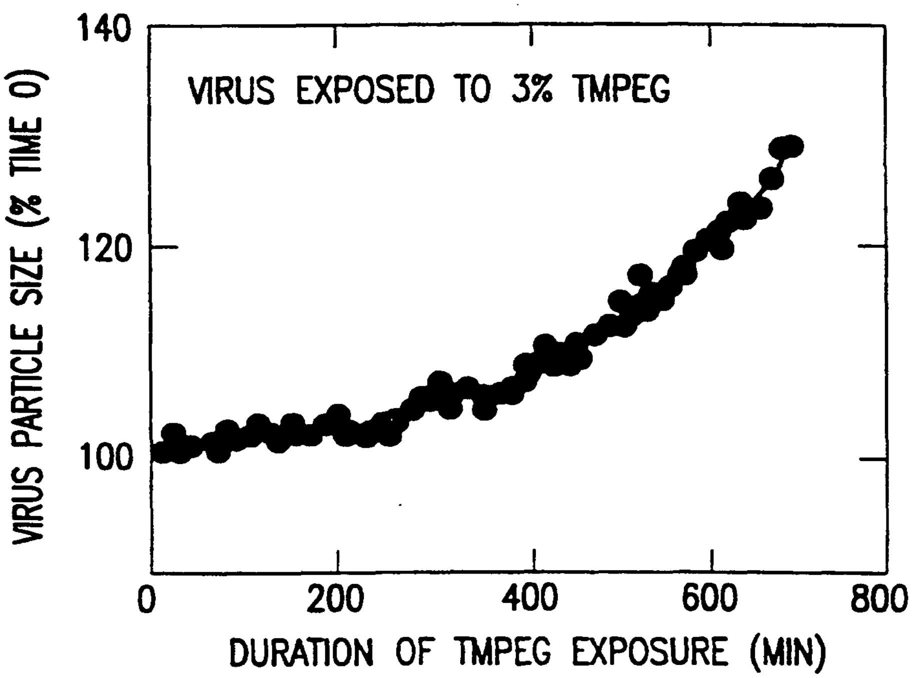

VIRUS EXPOSED TO 3% TMPEG
VIRUS PARTICLE SIZE (% TIME 0)
140
120
100
0
200
400
600
800
DURATION OF TMPEG EXPOSURE (MIN)

FIG.3D

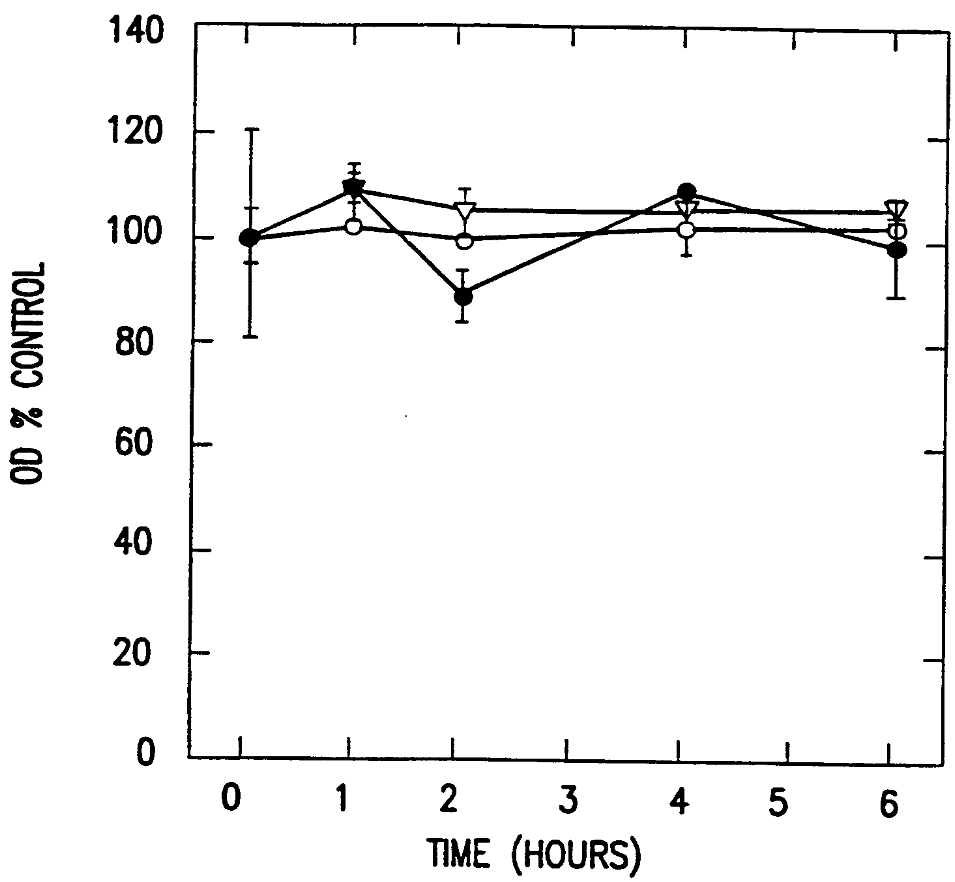


FIG.4

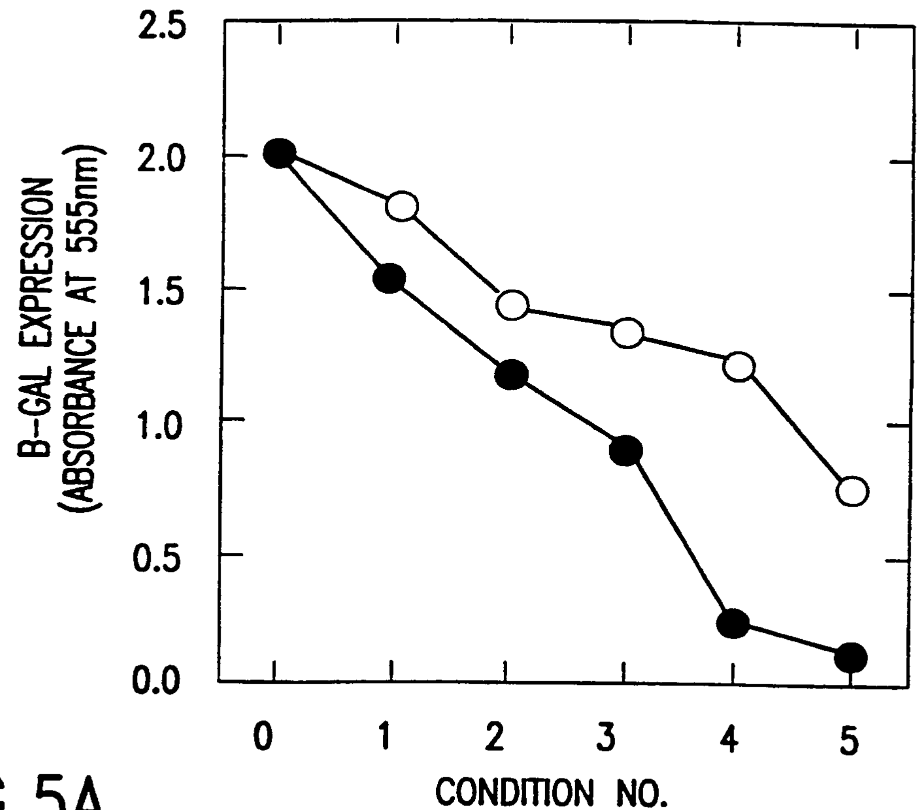

2.5
2.0
1.5
1.0
0.5
0.0
B-GAL EXPRESSION
(ABSORBANCE AT 555nm)
0
1
2
3
4
5
CONDITION NO.

FIG.5A

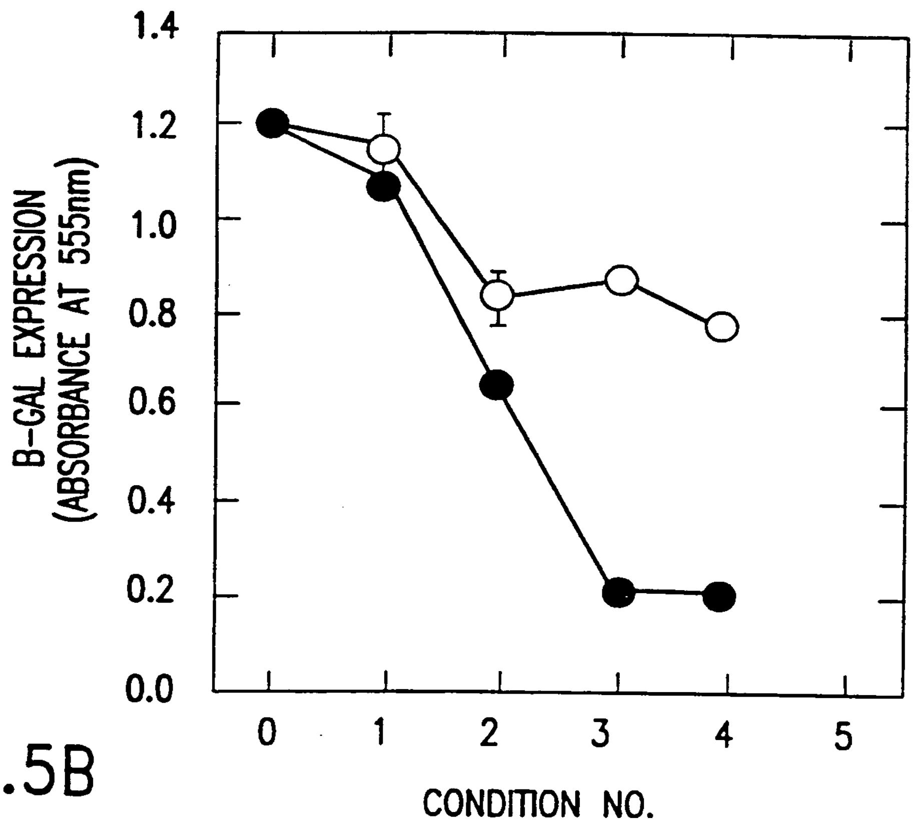

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
B-GAL EXPRESSION
(ABSORBANCE AT 555nm)
0
1
2
3
4
5
CONDITION NO.

FIG.5B

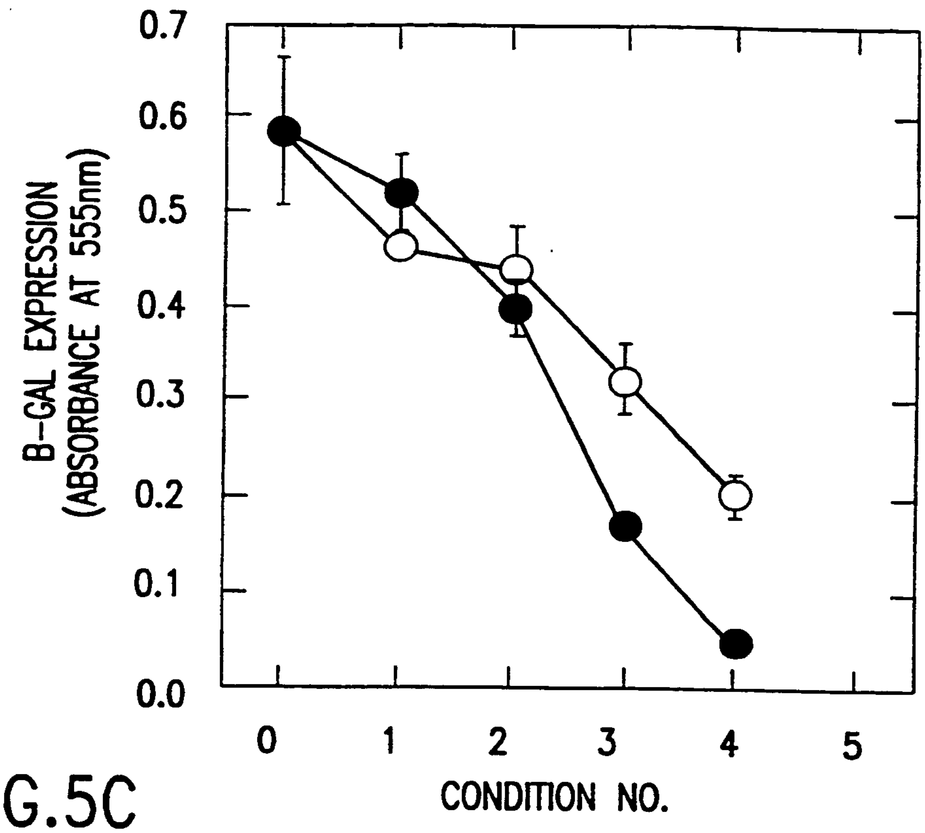

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
B-GAL EXPRESSION
(ABSORBANCE AT 555nm)
0
1
2
3
4
5
CONDITION NO.

FIG.5C

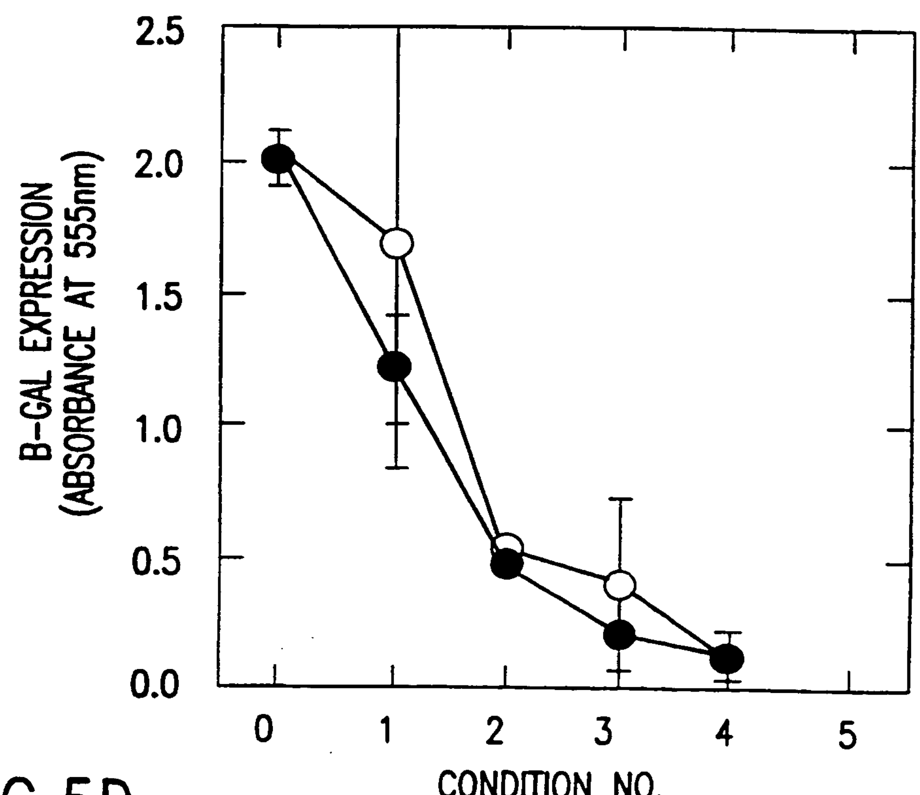

2.5
2.0
1.5
1.0
0.5
0.0
B-GAL EXPRESSION
(ABSORBANCE AT 555nm)
0
1
2
3
4
5
CONDITION NO.

FIG.5D

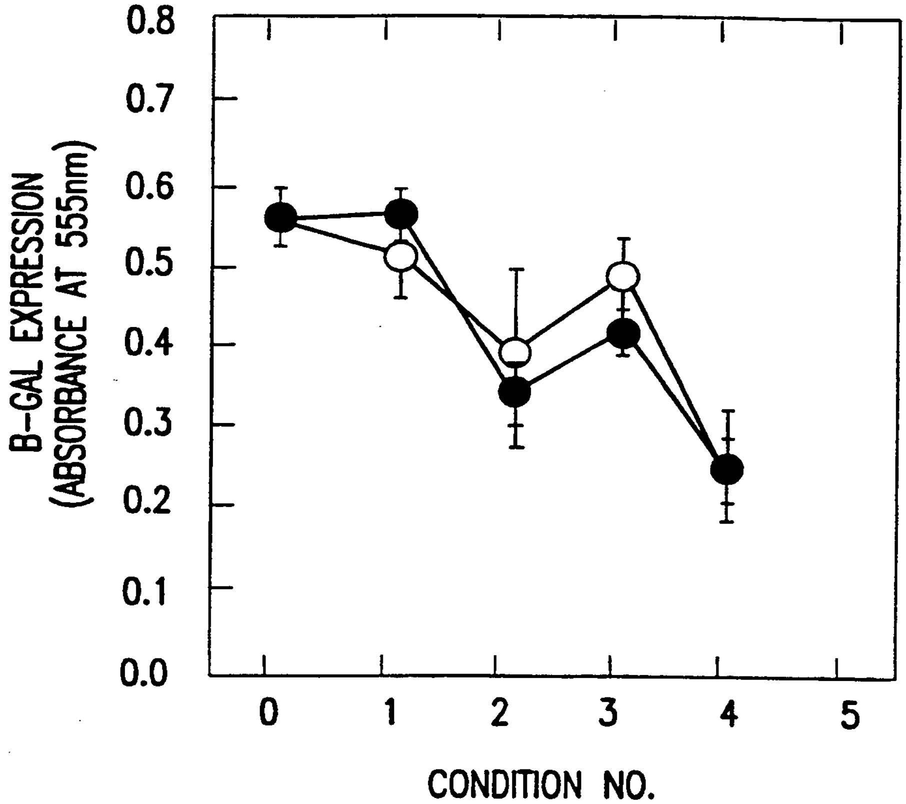

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
B-GAL EXPRESSION
(ABSORBANCE AT 555nm)
0
1
2
3
4
5
CONDITION NO.

FIG.5E

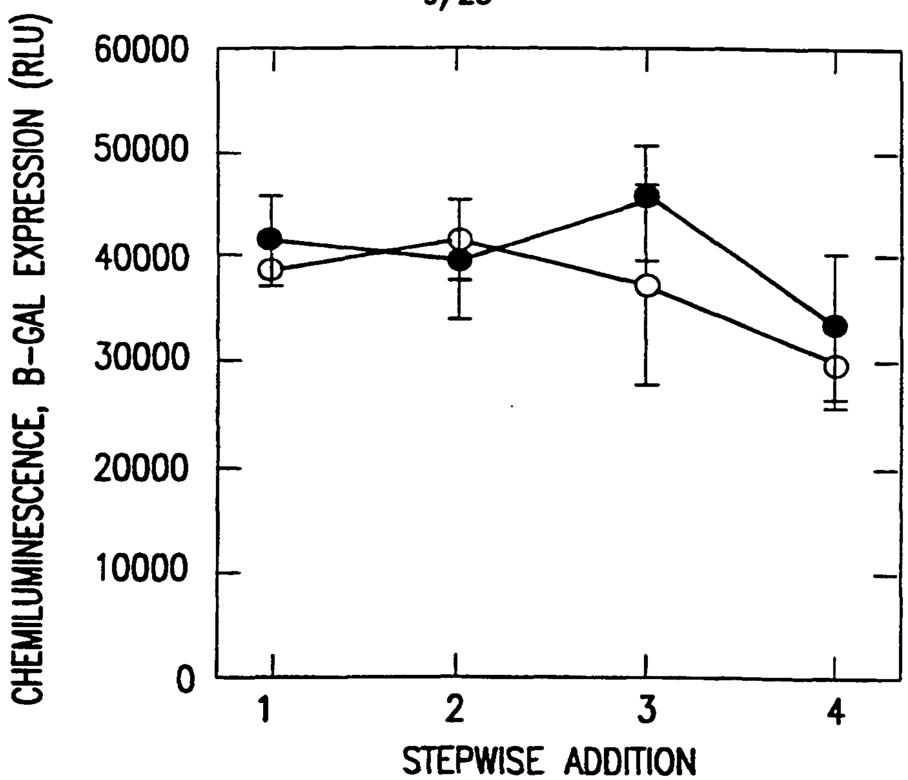

CHEMILUMINESCENCE, B-GAL EXPRESSION (RLU)
60000
50000
40000
30000
20000
10000
0
1
2
3
4
STEPWISE ADDITION

FIG.6A

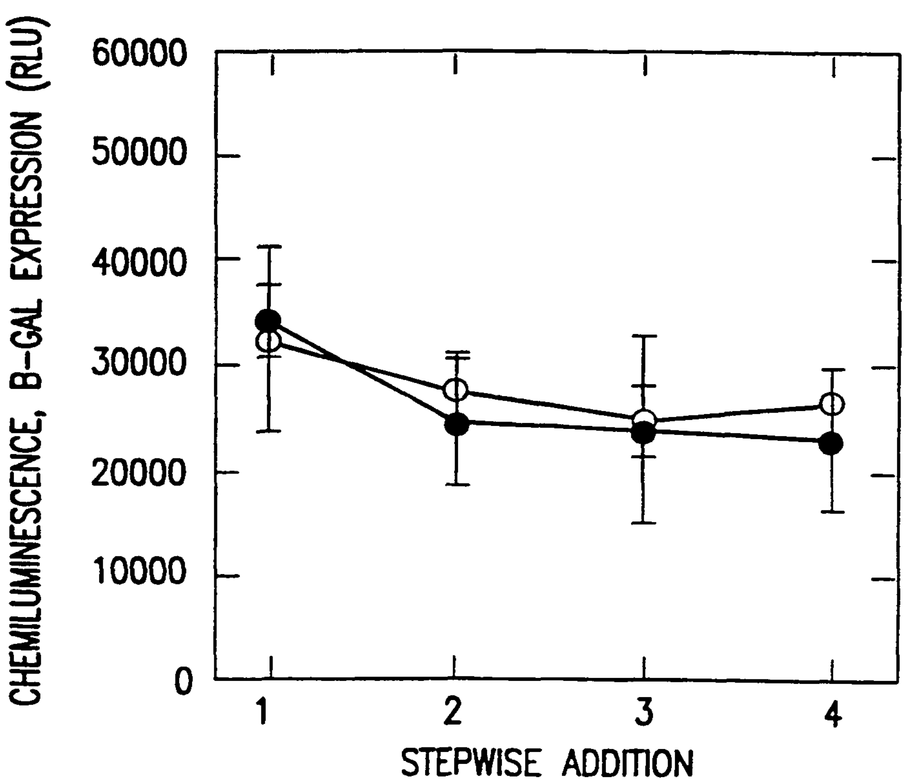

CHEMILUMINESCENCE, B-GAL EXPRESSION (RLU)
60000
50000
40000
30000
20000
10000
0
1
2
3
4
STEPWISE ADDITION

FIG.6B

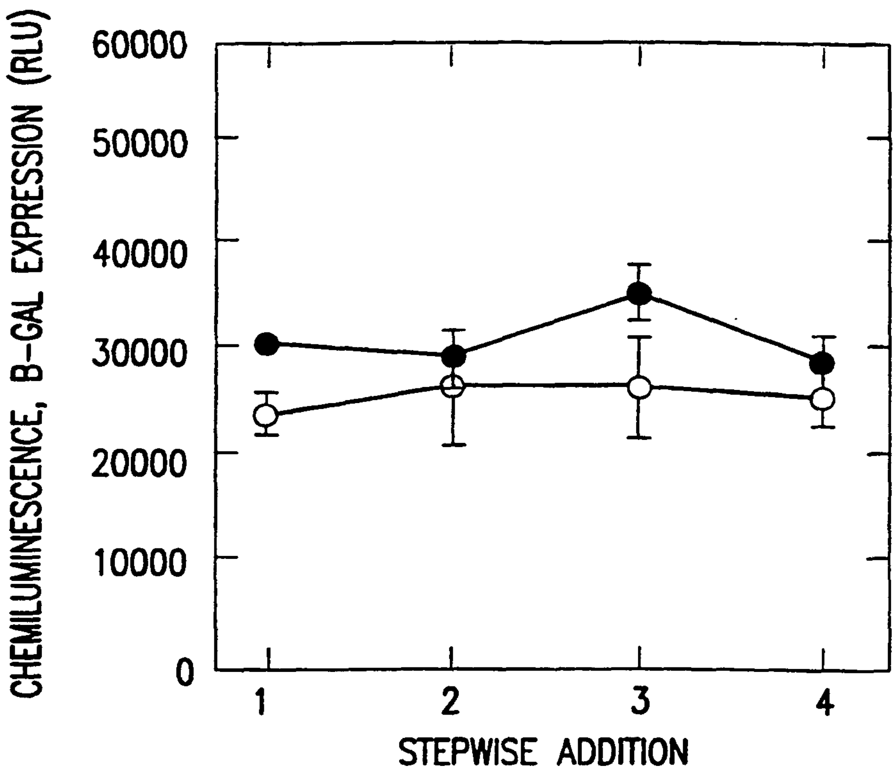

CHEMILUMINESCENCE, B-GAL EXPRESSION (RLU)
60000
50000
40000
30000
20000
10000
0
1
2
3
4
STEPWISE ADDITION

FIG.6C

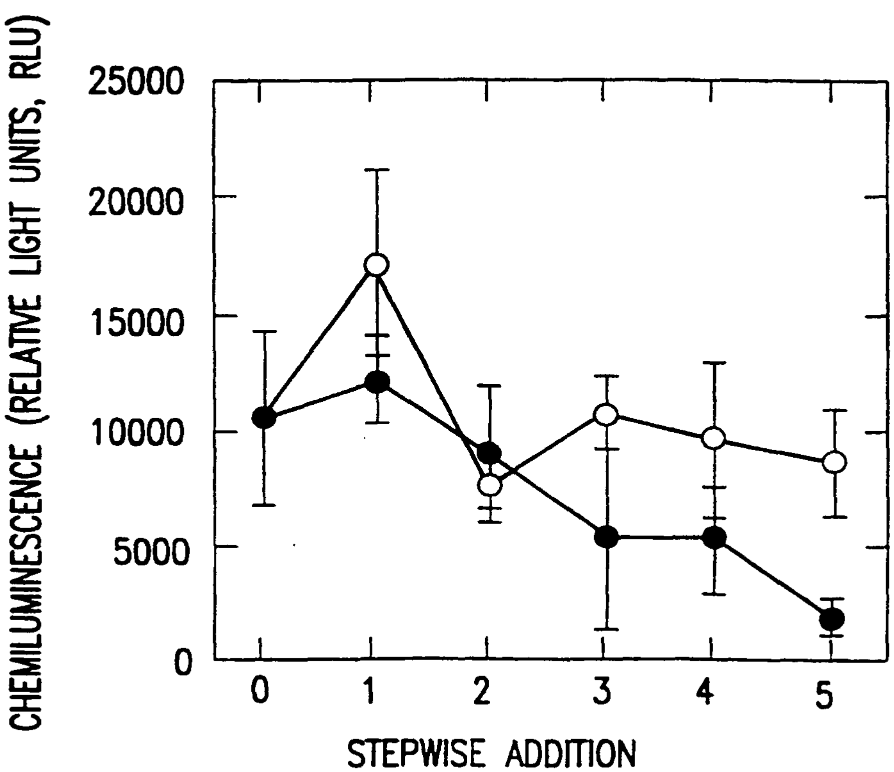

CHEMILUMINESCENCE (RELATIVE LIGHT UNITS, RLU)
25000
20000
15000
10000
5000
0
0
1
2
3
4
5
STEPWISE ADDITION

FIG.7A

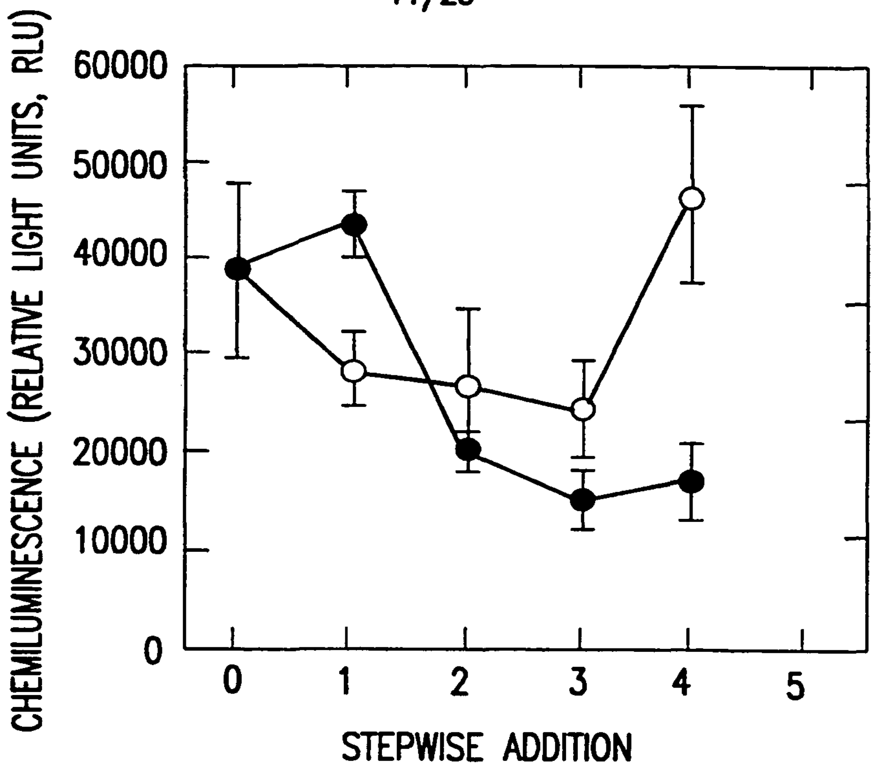

CHEMILUMINESCENCE (RELATIVE LIGHT UNITS, RLU)
60000
50000
40000
30000
20000
10000
0
0
1
2
3
4
5
STEPWISE ADDITION

FIG.7B

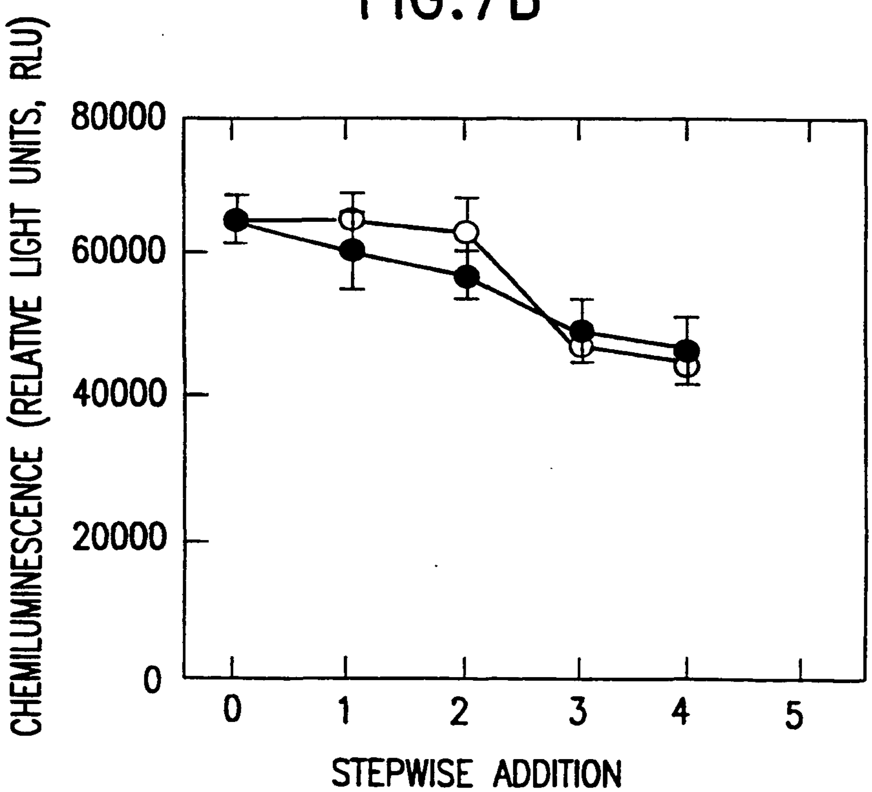

CHEMILUMINESCENCE (RELATIVE LIGHT UNITS, RLU)
80000
60000
40000
20000
0
0
1
2
3
4
5
STEPWISE ADDITION

FIG.7C

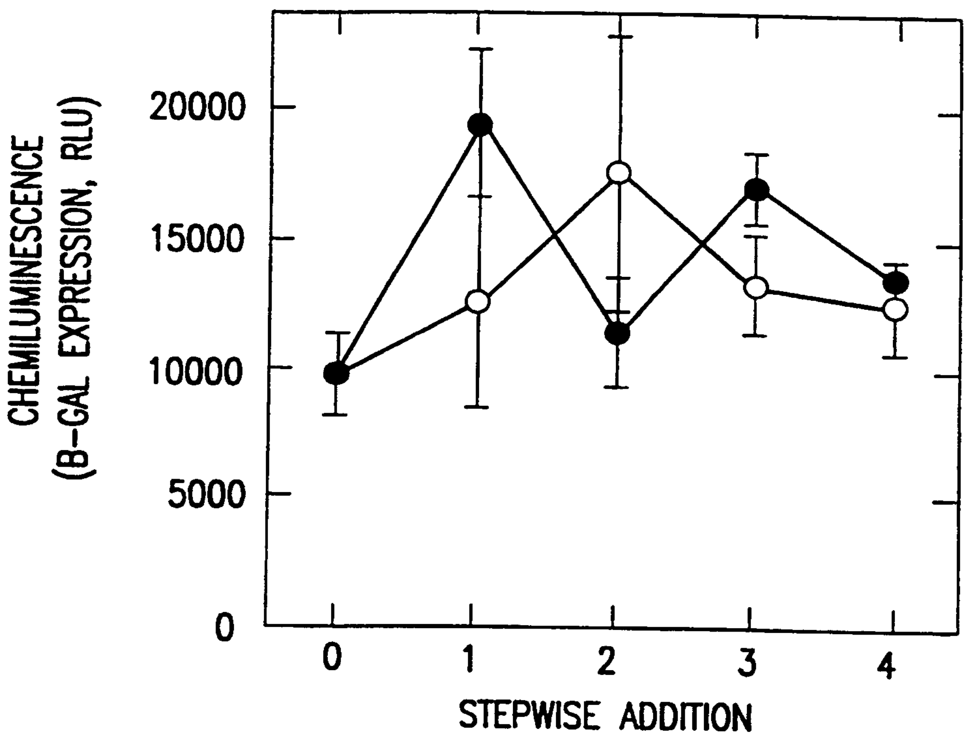

CHEMILUMINESCENCE (B-GAL EXPRESSION, RLU)
20000
15000
10000
5000
0
0
1
2
3
4
STEPWISE ADDITION

FIG.8A

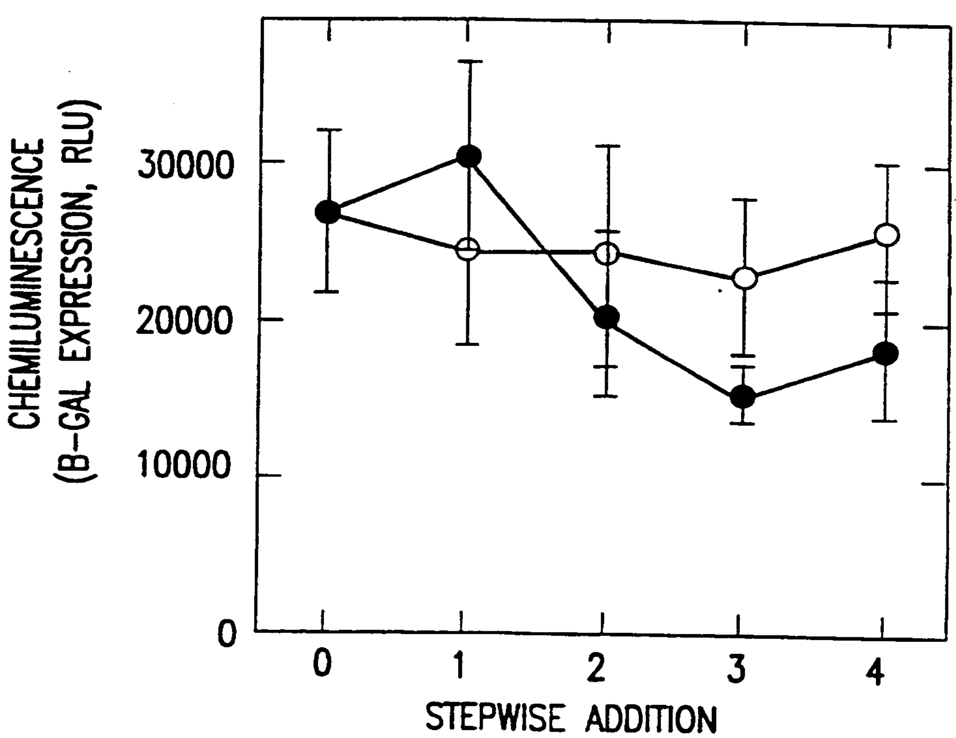
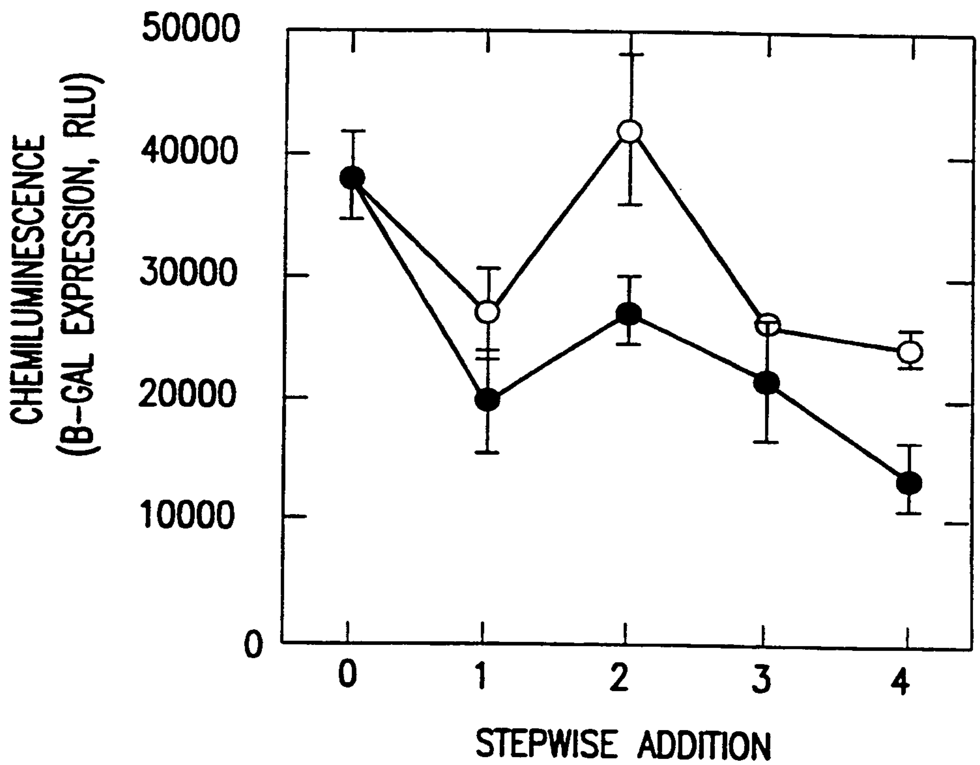

CHEMILUMINESCENCE (B-GAL EXPRESSION, RLU)
30000
20000
10000
0
0
1
2
3
4
STEPWISE ADDITION

FIG.8B



50000
40000
30000
20000
10000
0
CHEMILUMINESCENCE (B-GAL EXPRESSION, RLU)
0
1
2
3
4
STEPWISE ADDITION

FIG.8C

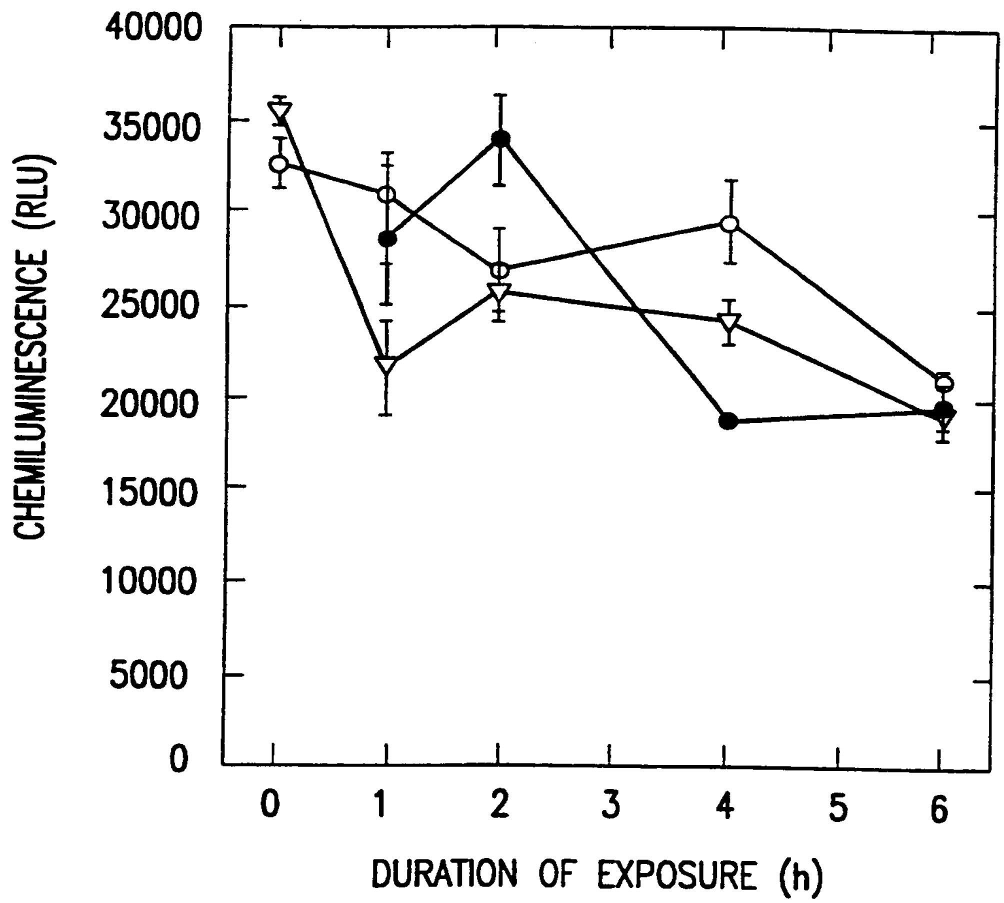

40000
35000
30000
25000
20000
15000
10000
5000
0
CHEMILUMINESCENCE (RLU)
0
1
2
3
4
5
6
DURATION OF EXPOSURE (h)

FIG.9

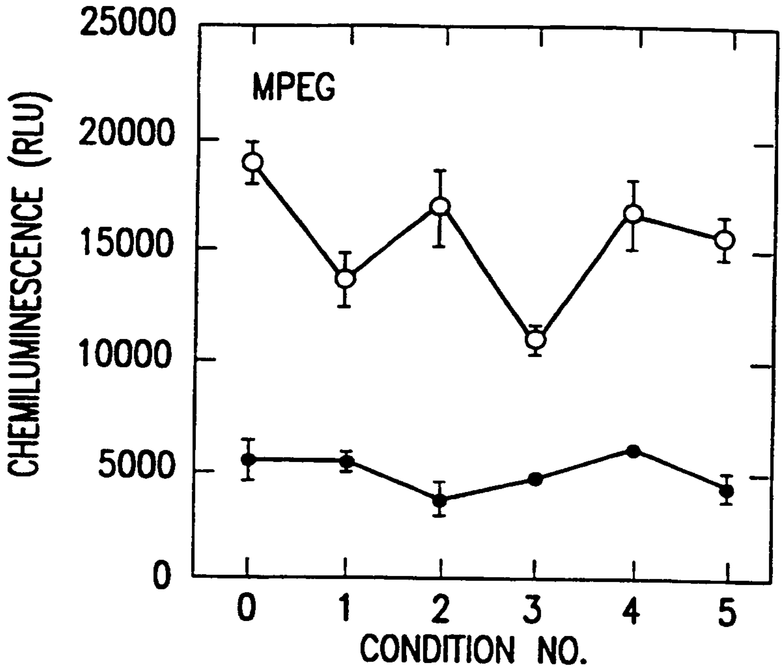


FIG.10A

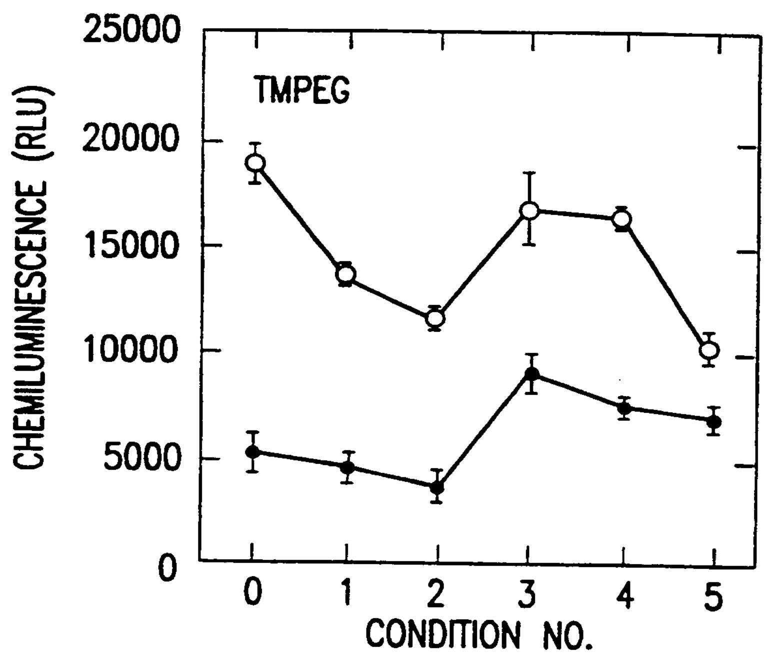


FIG.10B

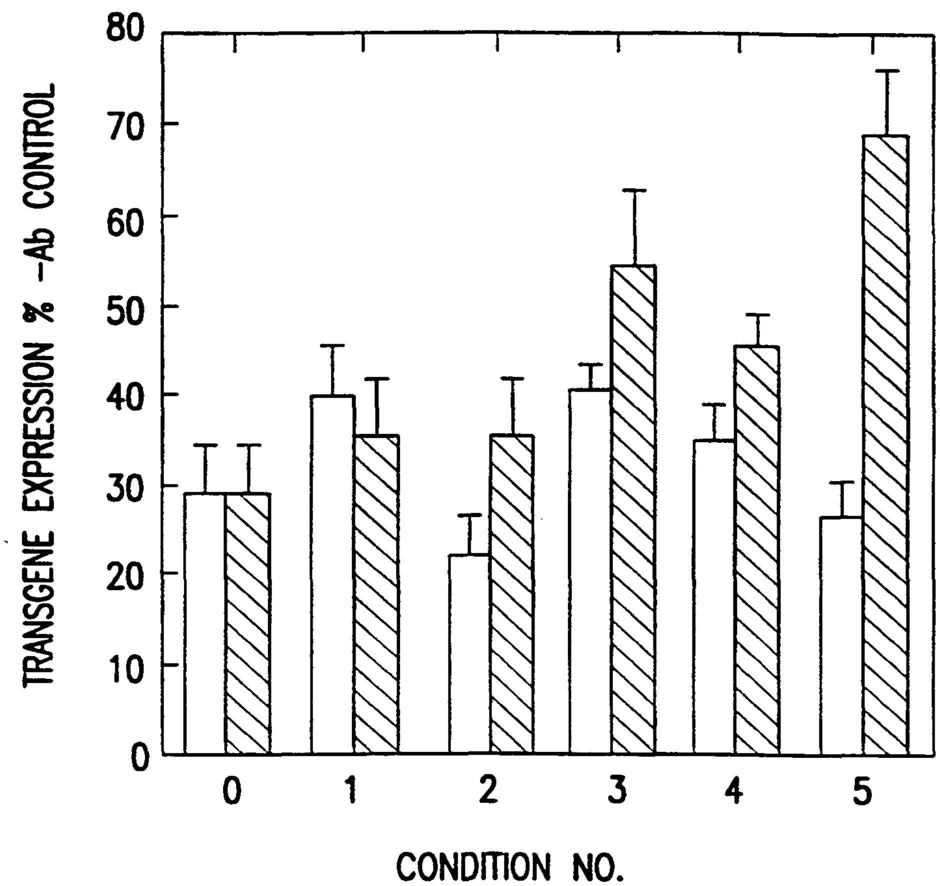

80
70
60
50
40
30
20
10
0
TRANSGENE EXPRESSION % -Ab CONTROL
0
1
2
3
4
5
CONDITION NO.

FIG.10C

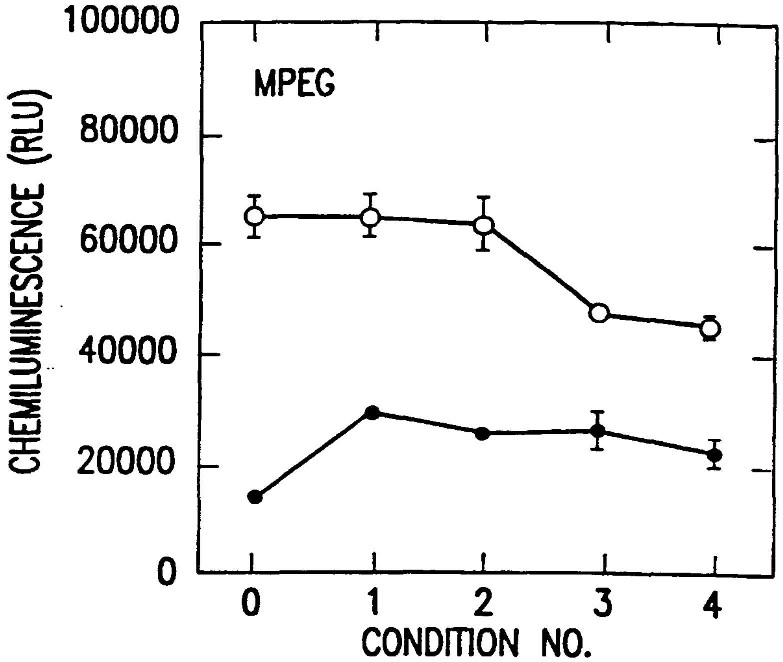


FIG.11A

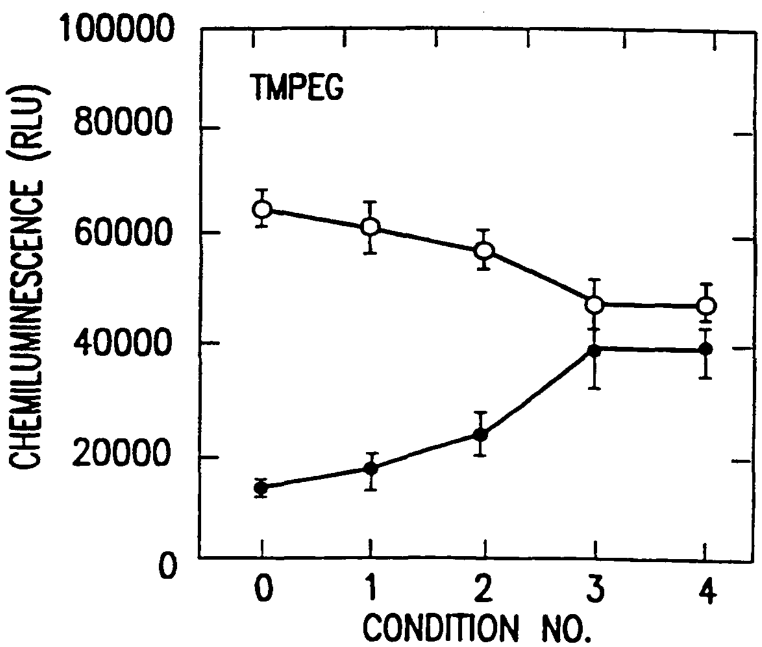


FIG.11B

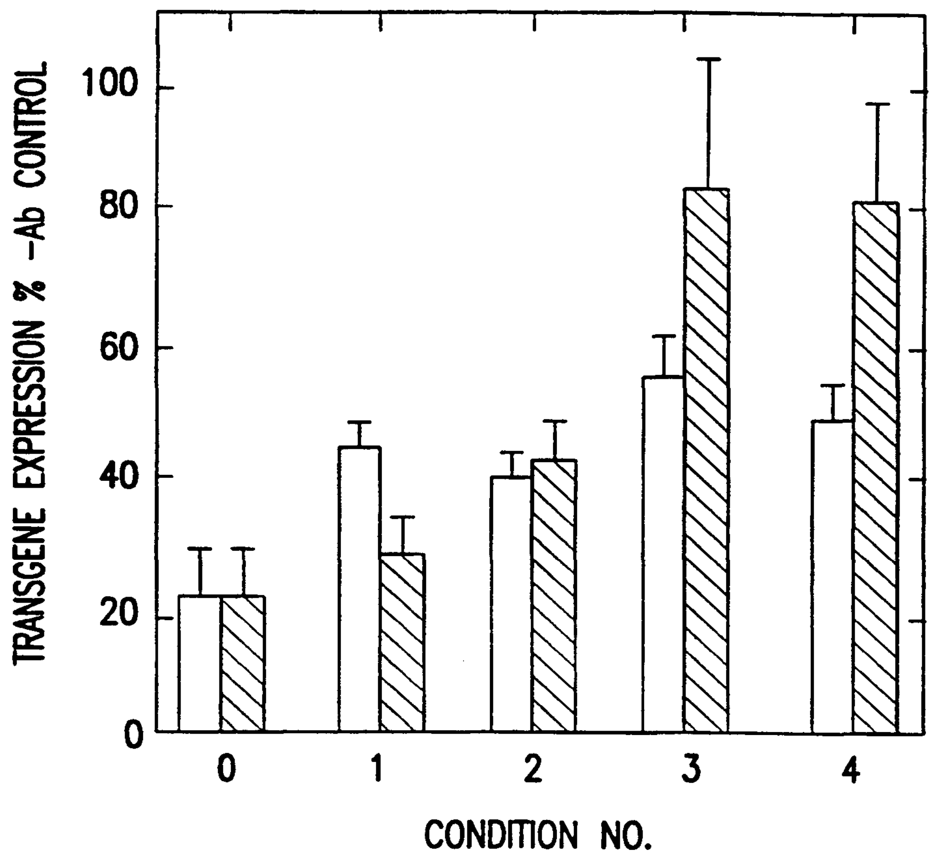

TRANSGENE EXPRESSION % -Ab CONTROL
100
80
60
40
20
0
0
1
2
3
4
CONDITION NO.

FIG.11C

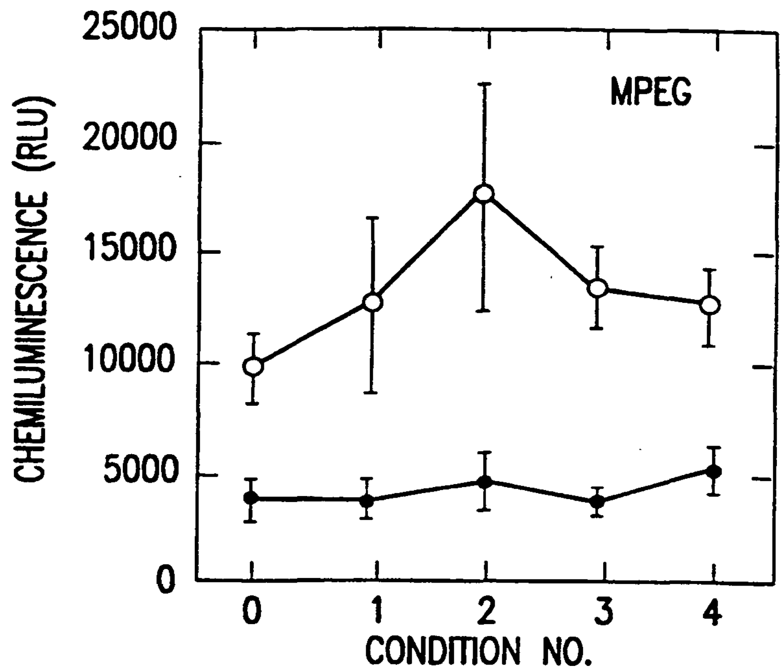


FIG.12A

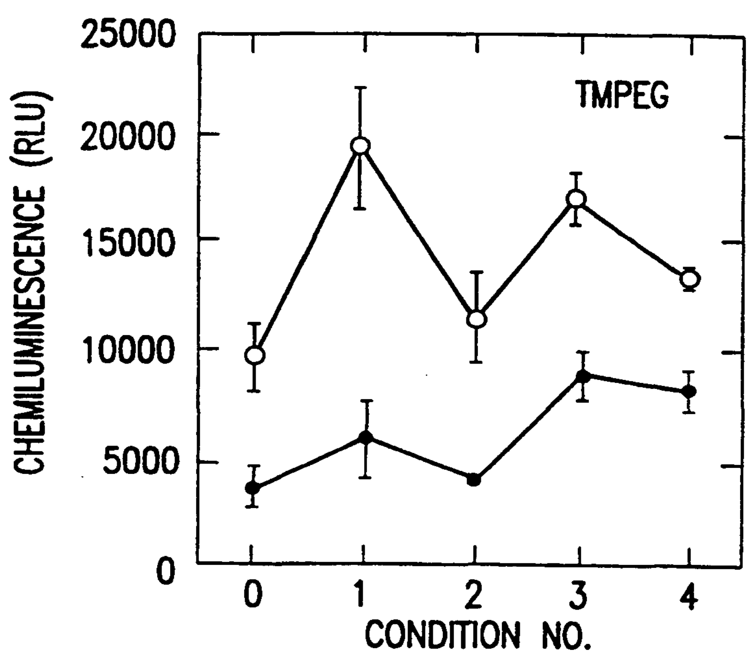


FIG.12B

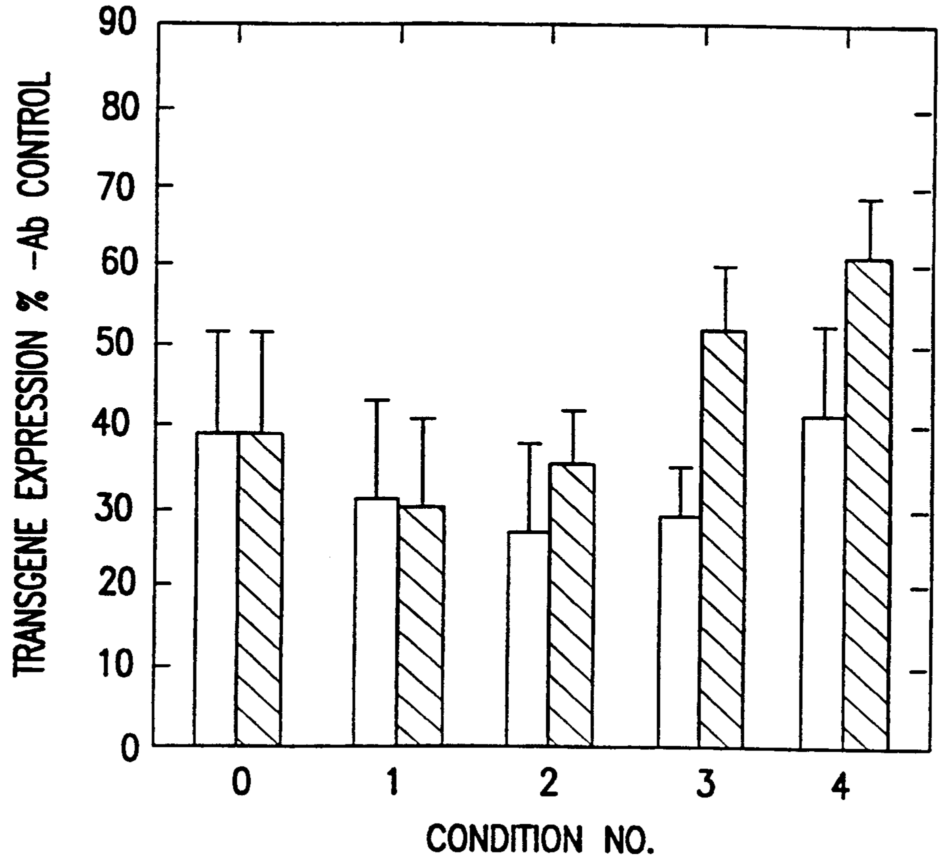

TRANSGENE EXPRESSION % -Ab CONTROL
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
CONDITION NO.

FIG.12C

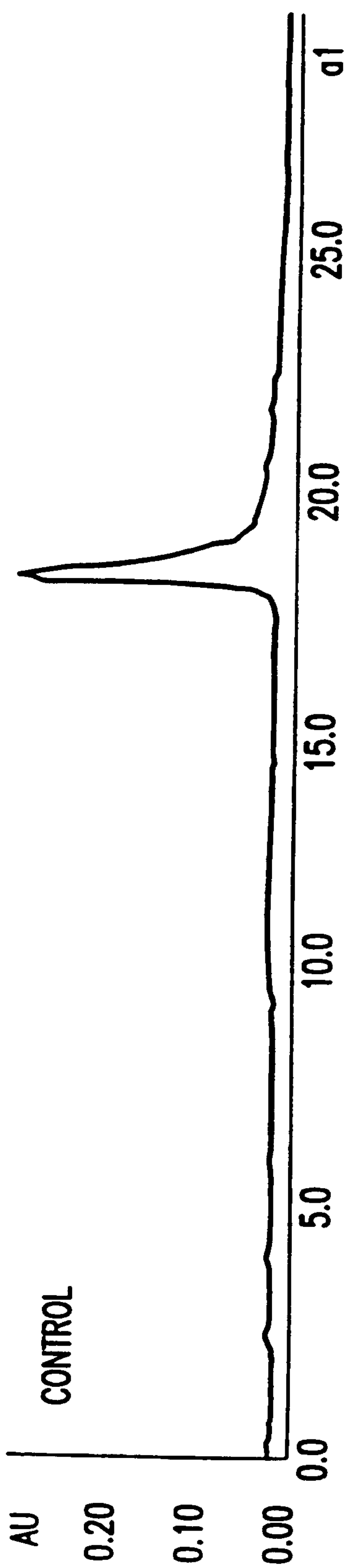


FIG. 13A

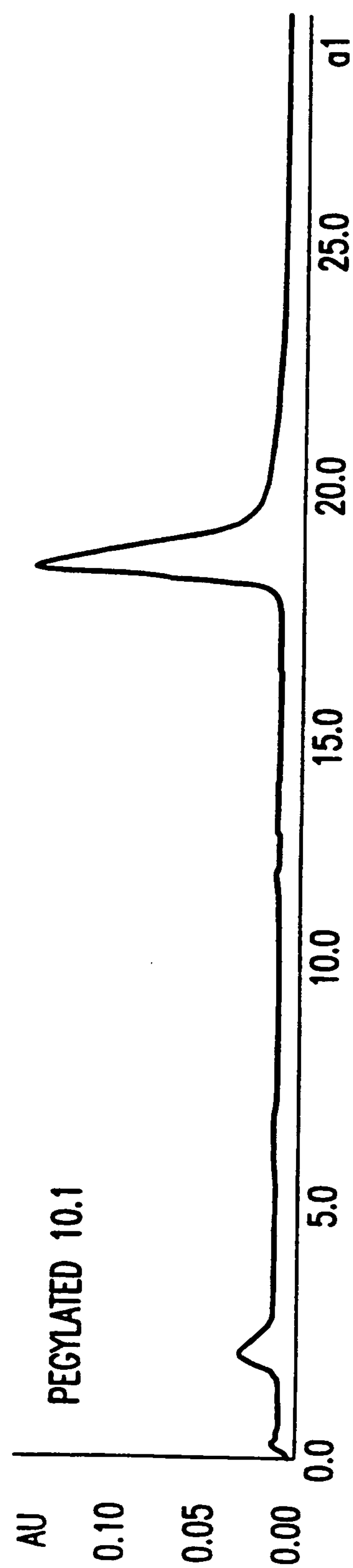


FIG. 13B

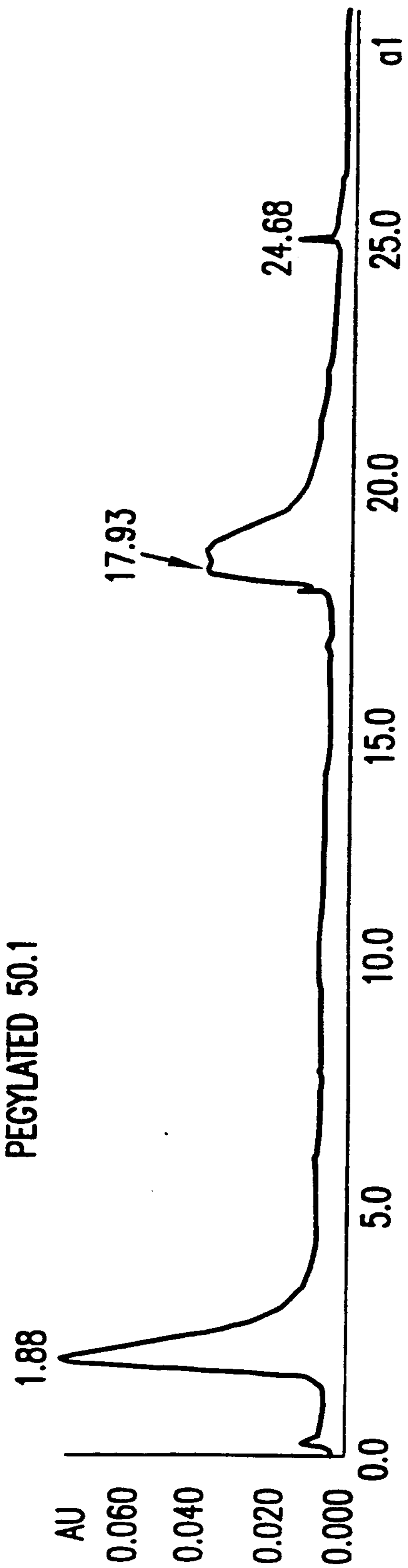

PEGYLATED 50.1
AU
0.060
0.040
0.020
0.000
0.0
5.0
10.0
15.0
20.0
25.0
1.88
17.93
24.68

FIG. 13C

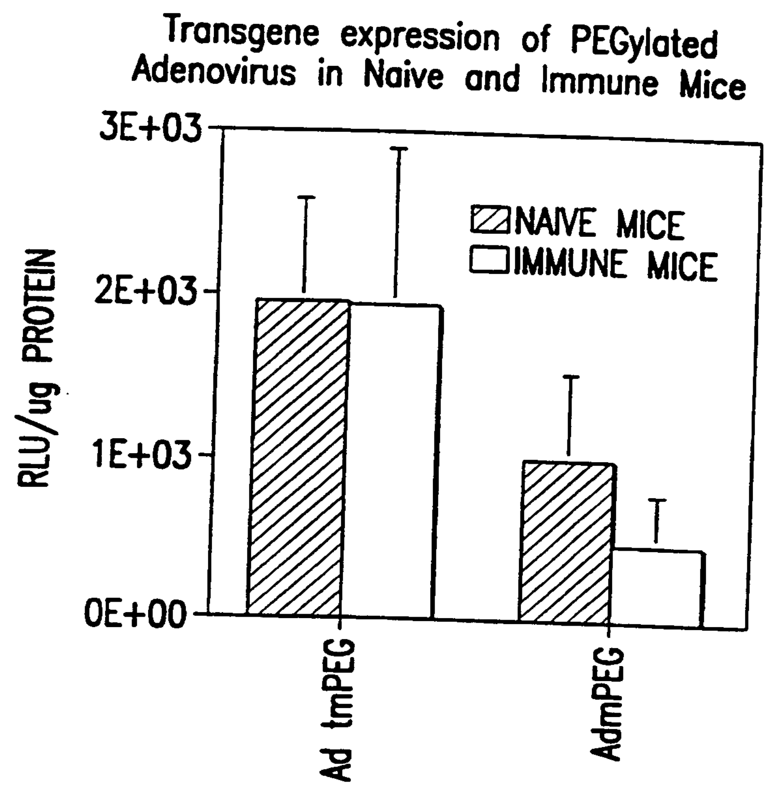

Transgene expression of PEGylated Adenovirus in Naive and Immune Mice
3E+03
2E+03
1E+03
0E+00
RLU/ug PROTEIN
NAIVE MICE
IMMUNE MICE
Ad tmPEG
AdmPEG

FIG. 14

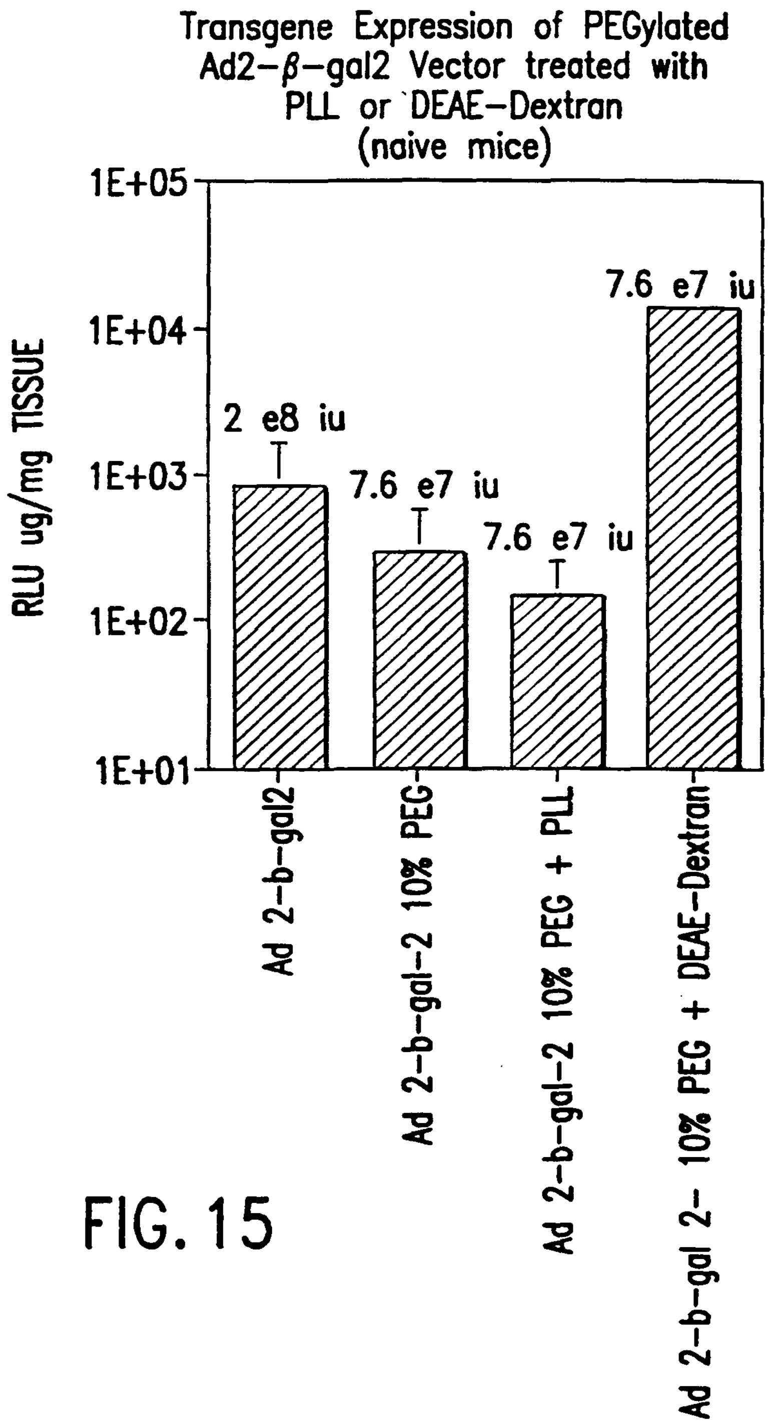

Transgene Expression of PEGylated
Ad2-β-gal2 Vector treated with
PLL or DEAE-Dextran
(naive mice)
1E+05
1E+04
1E+03
1E+02
1E+01
RLU ug/mg TISSUE
2 e8 iu
7.6 e7 iu
7.6 e7 iu
7.6 e7 iu
Ad 2-b-gal2
Ad 2-b-gal-2 10% PEG
Ad 2-b-gal-2 10% PEG + PLL
Ad 2-b-gal 2- 10% PEG + DEAE-Dextran

FIG. 15

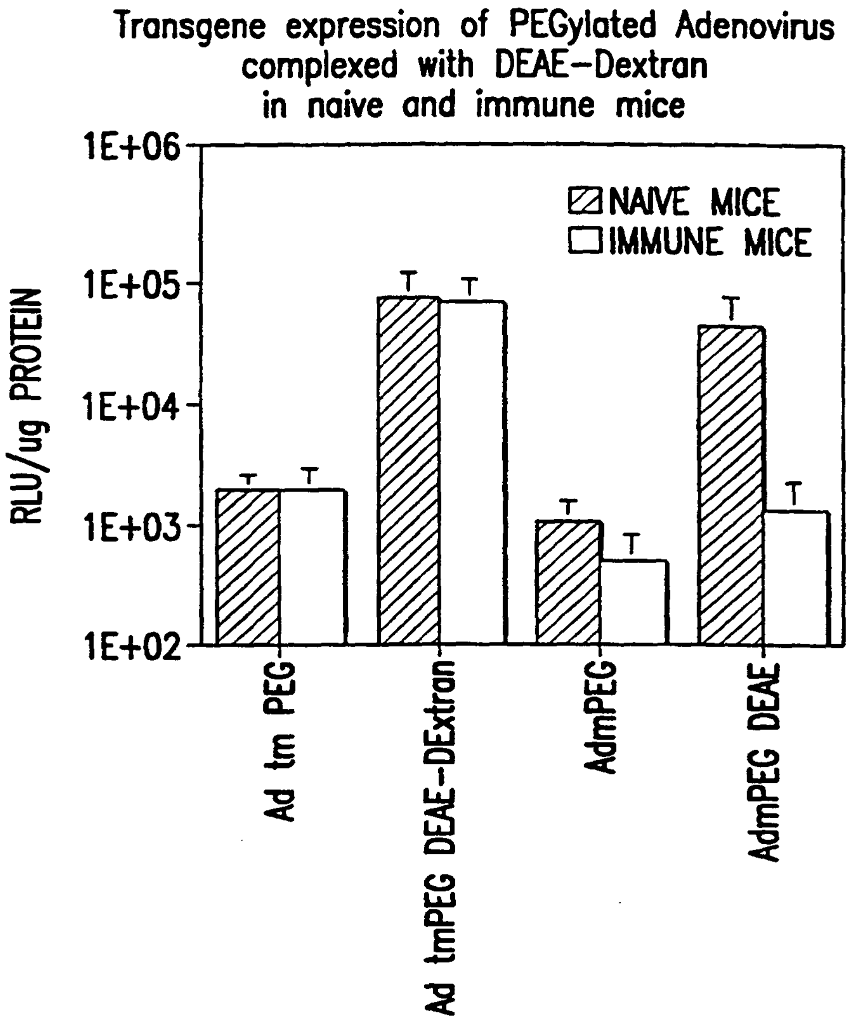

Transgene expression of PEGylated Adenovirus complexed with DEAE-Dextran in naive and immune mice
1E+06
1E+05
1E+04
1E+03
1E+02
RLU/ug PROTEIN
NAIVE MICE
IMMUNE MICE
Ad tm PEG
Ad tmPEG DEAE-DExtran
AdmPEG
AdmPEG DEAE

FIG. 16

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/19162

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/86 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 21036 A (VIAGENE INC) 11 July 1996 (1996-07-11) page 2, line 13-18 page 3, line 17-29 page 14, line 12 example 1	1-10, 18-22
X	CHILLON M ET AL: "Adenovirus complexed with polyethylene glycol and cationic lipid is shielded from neutralizing antibodies in vitro." GENE THERAPY, (1998 JUL) 5 (7) 995-1002. , XP002078070 abstract page 995, right-hand column, line 28 -page 996, left-hand column, line 40	1-10, 18-22

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 December 1999	16/12/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Covone, M

1

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	AL FASBENER ET AL: "Complexes of Adenovirus with polycationic polymers and cationic Lipids Increase the efficiency of Gene Transfer in Vitro and in Vivo" JOURNAL OF BIOLOGICAL CHEMISTRY,US,AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, vol. 272, no. 10, page 6479-6489 XP002078069 ISSN: 0021-9258 abstract page 6488, right-hand column, paragraph 1 ---	1-10, 18-22
A	US 5 521 291 A (BIRNSTIEL MAX L ET AL) 28 May 1996 (1996-05-28) figure 1 ---	11-17
P,X	O'RIORDAN C R ET AL: "PEGylation of adenovirus with retention of infectivity and protection from neutralizing antibody in vitro and in vivo." HUMAN GENE THERAPY, (1999 MAY 20) 10 (8) 1349-58. , XP000857510 abstract page 1356, right-hand column, paragraph 2 -page 1357, left-hand column, paragraph 2 figure 4 ---	1-10, 18-22
P,A	WO 98 44143 A (DELGADO CHRISTINA ;FRANCIS GILLIAN E (GB); POLYMASC PHARMACEUTICAL) 8 October 1998 (1998-10-08) page 2, line 29 -page 3, line 16 -----	1-22

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interr ıal Application No
PCT/US 99/19162

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
WO 9621036	A	11-07-1996	AU	4690596	A	24-07-1996
US 5521291	A	28-05-1996	AU	669335	B	06-06-1996
			AU	2593292	A	03-05-1993
			CA	2114800	A	15-04-1993
			CN	1071457	A	28-04-1993
			WO	9307282	A	15-04-1993
			EP	0535576	A	07-04-1993
			EP	0606280	A	20-07-1994
			FI	941473	A	30-03-1994
			HU	71322	A	28-11-1995
			HU	9500695	A	29-01-1996
			JP	7500009	T	05-01-1995
			MX	9205226	A	01-03-1993
			NO	941155	A	29-03-1994
			NZ	244291	A	26-07-1995
			ZA	9206767	A	11-06-1993
WO 9844143	A	08-10-1998	AU	6881798	A	22-10-1998